Factors Affecting Growth of the Arbuscular Mycorrhizal Fungal Mycelium

By

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For my Grandfather

Laurence Hubert Coulson
(BSc FRSC)
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Without whose wide-ranging and intelligent
interest in science, this may never have been written

Declaration

I hereby declare that this thesis has been composed by myself, and that the work of which it is a record is my own. It has not been accepted in any previous application for a degree. All quotations have been distinguished by quotation marks, and all sources of information have been specifically acknowledged by means of references.

May, 1997 Mary-Louise Ralph

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Summary

Arbuscular mycorrhizal relationships link plant root systems and zygomycetous fungi from the family Glomales in a symbiotic association which provides a carbon source to the fungus and benefits to the plant in terms of nutrient and water acquisition, and disease resistance. Root system colonisation is characterised by the formation of intracellular hyphae and internal fungal structures, the arbuscles and vesicles. Colonisation occurs after root penetration by fungal hyphae originating from either spores or extra-radical hyphae. The extra-radical hyphae, which form the fungal mycelium in the soil and have a primary role in host plant nutrition, are a largely neglected feature of the symbiosis due to the difficulties involved in their study.

In the present work, methodologies were developed to allow *in vitro* studies of extra-radical hyphae and thus circumvent problems associated with the inaccessibility and opacity of the soil environment. Root pieces colonised by the arbuscular mycorrhizal (AM) fungus *Glomus etunicatum* Becker & Gerdemann (S329, INVAM, USA) were inoculated onto dialysis membrane overlying transparent high purity agarose gel. This restricted the hyphae to a two-dimensional growth form which facilitated observation using microscopy and image analysis techniques, and enabled morphological measurements to be taken without the added complexity of three dimensional growth.

The two major influences on AM fungal growth are those of soil and of plant origin. The present work studied the effects of plant related factors on AM fungal hyphae. Host and non-host plant factors, and derivatives of these, were found to influence hyphal growth as characterised both by length measurements and morphological parameters. Host root exudates reduced hyphal growth, apparently as a result of changes in the overall distribution of hyphae within the mycelium, as measured by fractal dimension (FD). Non-host exudates and plant flavonoid compounds also decreased hyphal growth, but this result could not be attributed to changes in either branching or mycelial organisation. Root extracts of host and non-host plants did not significantly affect hyphal length, but did affect mycelial morphology. Although host root extracts appeared to have no effect on branching, they did alter hyphal distribution within the mycelium, as evidenced by an increase in FD. Non-host root extracts increased both branching and FD. Colonised host plant root extracts increased both hyphal length and branching, but had no significant effect on hyphal distribution within the mycelium.

Results are discussed in the context of previous work carried out on germ tube hyphae of AM fungi from spore inoculum. Observations of extra-radical hyphae showed differential responses when compared with germ tube hyphae more commonly studied by previous authors. This indicates that different phases of the fungal life cycle respond in different ways to similar environmental influences.

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Chapter 1

1 General Introduction

1.1 Mycorrhizal Associations

The term mycorrhiza basically describes the intimate relationship between a plant root and a symbiotic fungus, an association which links the soil environment and the functional nutrient absorbing system of the plant. Mycorrhizas were first described, literally as "fungus roots", by Frank in 1885. Continuing attempts to define the term mycorrhiza more specifically, which often place emphasis on structure rather than function, have led to ambiguity over the precise nature of the relationship between the two organisms (Trappe, 1996). Although the term generally implies a mutualistic relationship, the balance between fungus and host may vary both with developmental stage and environment (Harley & Smith, 1983) and the importance of the structure-function relationship should be recognised in classification of a mycorrhizal association. Mycorrhizas can be reasonably defined as:

"the association between fungi and roots, or any other organ of higher plants involved in plant nutrient uptake from soil, usually considered a mutualistic symbiosis because of the highly interdependent, and commonly beneficial relationships established between both partners, in which the host plant receives mineral nutrients via the fungal mycelium (mycotrophism), while the heterotrophic fungus obtains carbon compounds from the host plant photosynthates" (Azcón-Aguilar & Bago, 1994 from Harley & Smith, 1983 and Harley, 1989).

Although they have been the subject of continued research, the role of mycorrhizas in both ecological and physiological context continues to present a scientific challenge. The most direct, and often dominant, effect of mycorrhizal fungi is due to their influence on nutrient uptake, and the subsequent translocation and delivery of nutrients to the host plant (George *et al.*, 1996). However, root colonisation by mycorrhizal fungi has a range of additional effects on plant growth, mediated via changes in root morphology, carbon distribution, water uptake and pathogen resistance (George *et al.*, 1996). The potential importance of mycorrhizas in these additional and diverse aspects of a plant's ability to grow and survive in a range of habitats is becoming increasingly recognised as a research area in addition to their already well-documented role in nutrient uptake (Reid, 1990).

1.2 Mycorrhizal Subgroups

Mycorrhizal symbioses fall into two major groupings, the ***ectomycorrhizas*** and the ***endomycorrhizas***. The ectomycorrhizas, which are found in tree species including members of the Pinaceae, Fagaceae and Betulaceae, encompass those symbioses in which the fungal partner does not penetrate the root cortical cells. Such

mycorrhizas form a characteristic mantle of hyphae around the root in conjunction with an inter-cellular network of mycelium within the root cortex termed the Hartig net. Most of the ectomycorrhizal fungi are basidiomycetes, although there are also several examples of ascomycetous and zygomycetous associations (Rhodes, 1980).

In contrast, the zygomycetous endomycorrhizal fungi grow both inter- and intra- cellularly within the root cortex, and the extra-radical hyphae extend out into the soil rather than forming a sheath around the root. The three endomycorrhizal subgroups are the ericaceous mycorrhizas, orchidaceous mycorrhizas and the arbuscular mycorrhizal fungi (AMF).

The majority of plant species are mycorrhizal, and 70% of species are host to the zygomycetous fungi which form arbuscular mycorrhizal associations (Francis & Read, 1995). The AMF are the most commonly occurring group and are an integral part of many plants, found on a vast taxonomic range as a result of associations formed by the single family Glomales (previously Endogonaceae).

1.3 Development and Impact of AM Colonisation

Initial root system infection occurs as a result of root penetration by hyphae which may arise either from spores or from extra-matrical hyphae of previously infected roots. Hyphal contact with a host root leads to the formation of an infection structure called an appressorium, followed by intracellular penetration of root cortical cells and the ultimate formation of internal fungal structures, the arbuscles and vesicles (Plate1). The arbuscles, which develop a short distance behind the apices of both inter- and intra-cellular hyphae, are highly branched, haustoria-like structures within the innermost cortical cells that surround the vascular cylinder, and have a probable function in nutrient transfer between the symbionts. Arbuscular development is initiated after hyphal penetration of the host cell by repeated dichotomous branching, and mature arbuscles typically occupy a large proportion of the host cell volume. The vesicles, which form as the infection ages, act as storage organs. Internal spread of the fungus in the cortex is followed by delayed proliferation of a hyphal network that develops outside the root and acts as a nutrient absorbing system. This cellular invasion is not damaging to the integrity of the cortical cells and is tolerated by the host despite repercussions in terms of plant metabolism and carbohydrate drain, ultimately exerting a positive influence on several aspects of plant physiology (Gianinazzi-Pearson & Gianinazzi, 1983). The AMF interact with both host plant and soil microflora, and mediate a bi-directional nutrient flow (Bethlenfalvay & Ames, 1987).

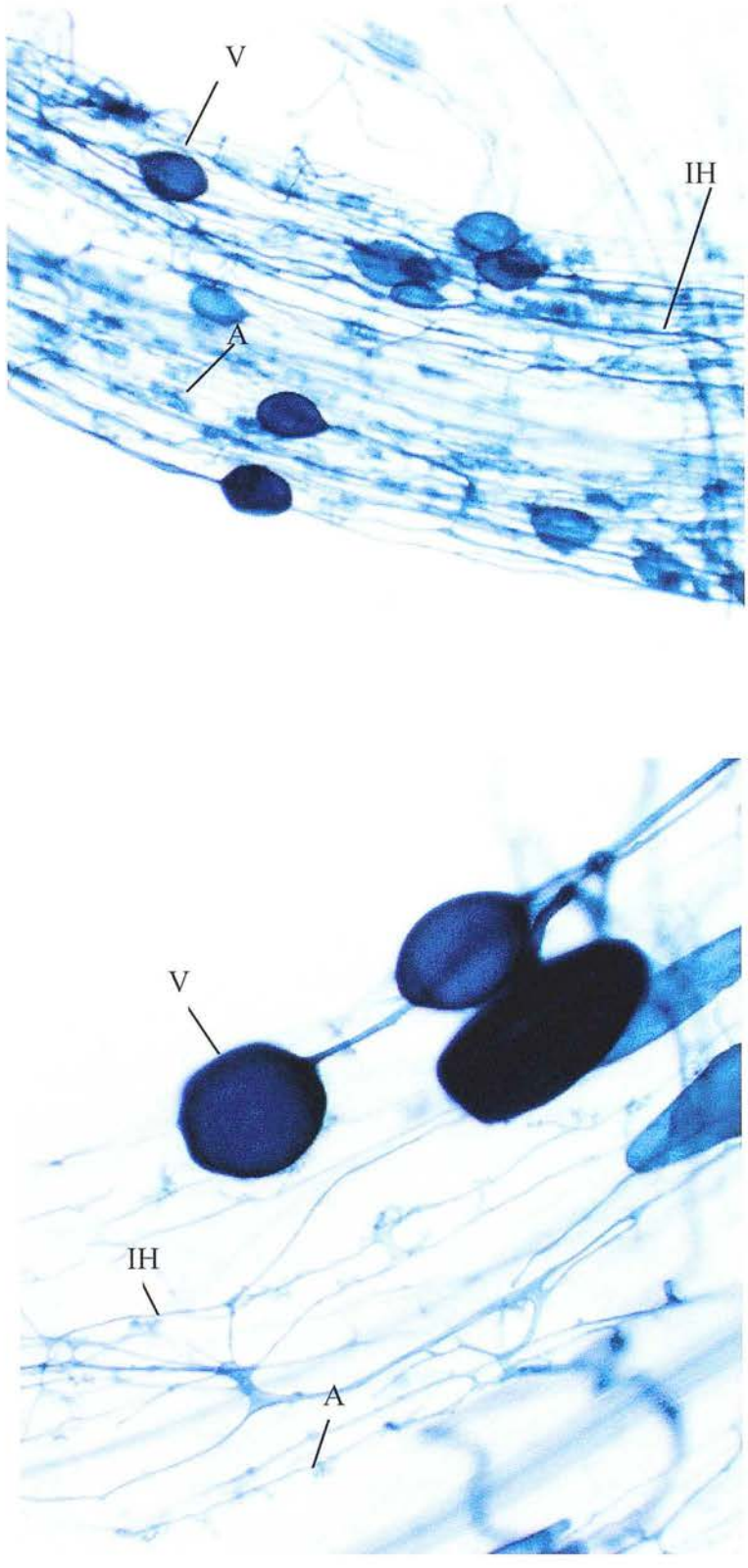


Plate 1: Arbuscular mycorrhizal colonisation showing internal hyphae (IH), vesicles (V) and arbuscles (A)

Mycorrhizal fungi exhibit unique carbon strategies which enable efficient coupling of soil mineralisation and nutrient uptake by the plant without the immobilisation of mineral nutrients that normally accompanies the consumption of carbon by soil bacteria and fungi (Reid, 1990). Carbon obtained from the host plant is rapidly converted to specific fungal compounds which cannot be readily utilised by the plant; this maintains a concentration gradient of photosynthate from the host to the fungus and therefore facilitates net movement to the heterotroph (Gianinazzi-Pearson & Gianinazzi, 1983). It is recognised that the mycorrhizal system provides a link between the root and the bulk soil which can promote the ability of mycorrhizal plants to acquire nutrients from the soil, primarily by increasing absorptive surface area. Any factors which increase surface area are therefore implicated. Hyphal diameter and length are of obvious importance in determination of basic uptake potential, but structural and spatial morphology will have a major impact on actual uptake. Distribution of this additional surface relative to the root is crucial. Hyphae immediately adjacent to a root surface will merely compete for diffusion-limited supplies of immobile nutrients such as phosphorus. Dispersed distribution away from the root lies outside the depletion zone allowing additional uptake and providing a net gain to the plant (Sanders & Tinker, 1971). Consideration must therefore be given to additional factors able to influence the surface area for absorption in mycorrhizal systems, such as the physical extent of the hyphal system, its functional longevity, absorbing power and ability to exploit nutrient rich microsites (Reid, 1990). The role of the external mycelium, and the relative ability of a particular mycorrhizal species to take up phosphorus and other nutrients, thus depends not only on its size and rate of spread, but also on its uptake and translocation capacities (Gianinazzi-Pearson & Gianinazzi, 1983; Jakobsen *et al.*, 1992).

The most recognised and well-documented aspect of mycorrhizal fungal associations is undoubtedly their effect on host plant phosphorus nutrition, which in deficient environments may rival the role of rhizobia in legume nitrogen nutrition (Sanders & Tinker, 1973).

Soil phosphorus levels are characterised by their heterogeneity of both form and quantity. Low ionic mobility leads to localised depletion and the slow mobilisation of concentrations (Tinker, 1975), increasing the likelihood of root depletion zones forming (Sanders & Tinker, 1971). Both mycorrhizal and non-mycorrhizal plants absorb phosphate from soil solution or forms in rapid equilibrium with solution. Neither roots nor hyphae appear able to solubilise non-labile inorganic phosphates, and until relatively recently it was also thought that neither were able to hydrolyse organic phosphates (Sanders & Tinker, 1971), thus implying that mycorrhizal and

non-mycorrhizal plants draw their phosphorus from the same source (Sanders & Tinker, 1973). The organic fraction makes up a large proportion of total soil phosphorus, accounting for up to 80% of total P in most agricultural soils (Tarafdar & Claassen, 1988). Its relative significance to plant nutrition has not been fully assessed and is a source of continuing debate (Kroehler & Linkins, 1991; Pant *et al*, 1994), largely because for organic compounds to become available to plants they must be hydrolysed to an inorganic form through the action of phosphatase enzymes. There is a mounting body of evidence to suggest that plants are able to access phosphorus from organic sources almost as efficiently as from inorganic sources through the action of their phosphatase enzymes, and that limitations to the utilisation of organic phosphorus lie with the availability of hydrolysable organic sources (Tarafdar & Claassen, 1988) not, as previously suggested, with an inability to hydrolyse organic phosphates *per se* (Sanders & Tinker, 1971). Thus, plant produced phosphatases probably also have a significant role in plant phosphorus nutrition.

Evidence suggests that the primary mechanism for enhancement of phosphorus uptake in mycorrhizal plants is largely attributable to the more dispersed and efficient root system provided by, and the uptake and translocation through, the extra-radical mycelium (Sanders & Tinker, 1971; Sanders & Tinker, 1973; Hattingh *et al.*, 1973; Rhodes & Gerdemann, 1975; Pearson & Tinker, 1975). Mass flow in soil solution is responsible for the transport of <1% of absorbed phosphate, implicating entry and sunsequent transport via mycorrhizal hyphae in the inflow of phosphorus to the roots of mycorrhizal plants (Sanders & Tinker, 1971). Mycorrhizal onion plants have been observed to grow more rapidly than non-mycorrhizal plants in moderately phosphorus deficient soils, and exhibit a phosphorus inflow four times that into non-mycorrhizal roots (Sanders & Tinker, 1973). As it is probable that the roots of non-mycorrhizal plants were absorbing phosphorus at approximately the maximum possible rate, the greater inflow to roots of mycorrhizal plants is most likely to be accounted for by uptake and transport via extra-radical hyphae (Sanders & Tinker, 1973). Hyphae are able to grow more closely to soil colloids, and are therefore likely to be more advantageously positioned than roots for soil phosphorus absorption.

Rhodes & Gerdemann (1975) showed that *Glomus fasciculatum* (Thaxter) Gerd. & Trappe emend. Walker & Koske was able to extend the phosphorus uptake zone to a distance of at least seven centimetres from the root surface (growth was ultimately restricted by the size of the experimental chambers). This similarly implicated AMF in accessing phosphorus considerably outwith the one or two millimetre zone normally assumed to be the region of root phosphorus depletion. The release of such translocated phosphorus from fungus to host was demonstrated in a

Glomus mosseae (Nicol. & Gerd.) Gerdemann & Trappe/onion symbiosis (Pearson & Tinker, 1975). Similarly, Hattingh *et al.* (1973) showed that onion plants colonised by *Glomus mosseae* or *G. fasciculatum* received phosphorus in amounts not attributable to accumulation by diffusion. Mycorrhizal plants showed greater phosphorus accumulation than non-mycorrhizal plants, indicating that the mycelium was extending beyond the root zone of phosphorus removal, with concomitant plant growth benefits.

Whereas the relationship between AMF and host phosphorus nutrition is well documented, there has been relatively little attention directed at the influence of AMF on nitrogen availability. Both form and availability of nitrogen are important, as although the primary form of nitrogen available to plants is as ammonium, much soil nitrogen exists in bound organic form. Celery plants colonised by *Glomus mosseae* have been shown to derive more ^{15}N from both inorganic and organic sources of nitrogen than non-mycorrhizal plants (Ames *et al.*, 1983). This suggests that external hyphae of AMF can extend beyond the plant rhizosphere, take up nitrogen and transport it to the rooting zone, enabling host plant acquisition and incorporation into roots and shoots (Ames *et al.*, 1983). Hyphal transport of ^{15}N in cucumber by *Glomus intraradices* Schenk & Smith has also been demonstrated, indicating the ability of extra-radical hyphae to efficiently deplete the soil of inorganic nitrogen and implicating AMF in plant growth responses (Johansen *et al.*, 1992). *Glomus intraradices* was able to assimilate nitrogen from the plant growth medium, and results indicated that the fungus may have had a preference for ammonium over nitrate, probably because assimilation of ammonium requires less energy than that of nitrate (Johansen *et al.*, 1992).

Whether or not a mycorrhizal plant derives a net gain over a comparable non-mycorrhizal plant depends on the soil nitrogen content, the host and fungal requirement for nitrogen, and the form and placement of the nitrogen in the soil (Ames *et al.*, 1983). In agricultural soils, nitrate provides the primary nitrogen source. Nitrate is a mobile nutrient, readily diffusible to plant root systems, and AMF are thus unlikely to have a major impact on nitrogen acquisition where soil nitrate levels are high. However, under conditions where nitrification rates are low, ammonium predominates in the inorganic nitrogen pool. This is strongly adsorbed and therefore less mobile than nitrate, and AMF are thus likely to play a greater role in the transport of nitrogen to the root system (Johansen *et al.*, 1992). The more immobile an ion, the more relevant ion transport via mycorrhizal hyphae will become to the plant. Where competition for nitrogen is high, mycorrhizal colonisation may be either advantageous or necessary for plant growth (Johansen *et al.*, 1992).

On the basis of studies such as these, the mycorrhizal symbiosis is generally referred to as mutualistic, regardless of whether a functional analysis of the relationship has been carried out (Francis & Read, 1996). In de Bary's (1879) original definition, symbiosis was described as an intimate association of two organisms which could have negative as well as positive outcomes. In fact, although the majority of land plants in natural communities are recognised as being mycotrophic (Trappe, 1987), the functional basis of the relationship has been analysed in relatively few species (Francis & Read, 1996). The value of definitions based on structural rather than functional attributes is questionable, and experiments which simulate ecologically realistic conditions of seed germination in soil reveal a spectrum of fungal impacts of mutualism and antagonism. AMF in fact play a role in defining the ecological niches occupied by plants, and thus in the determination of plant community composition (Francis & Read, 1995). Some plants do not exhibit beneficial effects of colonisation, and in other cases negative effects have been observed (Francis & Read, 1996), particularly in conditions of high fertility (Kiernan *et al.*, 1983). It seems likely, for example, that turf incompatibility problems which occur in some species arise because of sensitivity to interference by AMF. There have been reports that colonisation can have adverse effects on plant species regarded as non-hosts, suggesting discrimination against some community species, and posing a determinant role for mycorrhiza in the distribution of individual plants and in the structure of natural and semi-natural plant communities. This has relevance to the concepts and nomenclature of mutualism and symbiosis, as well as to plant community structure (Francis & Read, 1995).

Symbiotic conditions are likely to represent a continuum from doubly enhancing to doubly detrimental impacts (Figure 1.1). Between these extremes associations exist which may be of benefit to neither partner, or in which only one of the partners benefits while the second either remains "neutral" or is negatively affected by the relationship. Although the mycorrhizal symbiosis has generally been considered as doubly enhancing, with widespread application of the term "mutualistic", it is already recognised that under particular conditions there may be no obvious benefit to one or other of the partners. For example, in highly fertile soils where nutrients are not limiting to the host plant, colonisation by AMF may inhibit plant growth. It has also been suggested that the fungus is likely to gain little benefit from an association with the roots of an achlorophyllous plant (Leake, 1994). Application of the term "mutualistic", inherently meaning "double benefit", may prove misleading when applied in the absence of experimental confirmation to mycorrhizal colonisation of plants under natural conditions. Evaluation of the symbiosis at the functional level is necessary for given plant species, as microscopic evidence of

colonisation provides justification for neither function nor mutualism (Francis & Read, 1995).

Partner	mutually enhancing (mycorrhizal symbiosis)					doubly detrimental
A	+	+	0	-	-	-
	Mutualism	Commensalism	Neutralism	Agonism	Amensalism	Competition
B	+	0	0	+	0	-

+ increase in potential fitness
0 potential fitness not affected
- decrease in potential fitness

Figure 1.1: The symbiotic continuum, defined as the potential fitness of two intimately associated species
(adapted by Francis & Read, 1995 from Lewis, 1985 & Bronstein, 1994)

Available concepts of symbiosis can readily accommodate situations where mycorrhizal fungi decrease survivorship and host fitness, but this response cannot be interpreted as mutualistic. In recent work, *Plantago lanceolata* L. showed a response to AMF typical of a mutualistic host plant, exhibiting significant increases in yield and vigour when mycorrhizal, but weak and with high mortality in the absence of inoculum. All other test species either from "non-host" families (Chenopodiaceae, Cruciferae, Polygonaceae) or families with a significant number of species regarded as mycorrhizal (Boraginaceae, Caryophyllaceae, Scrophulariaceae) showed converse responses with a strong growth inhibition in the presence of AMF, indicating a displacement to the negative end of the symbiotic continuum (Francis & Read, 1995). Differing results concerning the effect of AMF under experimental conditions highlight the need to clearly state the conditions under which these results were obtained, and to carry out comparisons with plants under more natural conditions. Negative results concerning mycorrhizal associations indicate the need to develop a broader view of the nature of the mycorrhizal symbiosis than that of mutualism, and highlight the importance of understanding both the potentially broad spectrum of responses that plants may show to infection by AMF, and those shown by AMF to the presence of host and non-host plants.

1.4 The Hyphal Growth Form

The hyphal growth form is characteristic of two groups of organisms, the filamentous prokaryotic actinomycetes and the filamentous fungi, in which it is most highly developed. Filamentous growth confers advantages over unicellular growth in two ways, through the bi-directional transport of materials along hyphae (unless restricted by septa) and through the maintenance of the surface area : volume ratio during growth (Prosser, 1983). Its most important feature is that it enables the fungus to explore and exploit new environments and substrates (Gooday, 1995).

Fungal hyphae are able to respond to a variety of environmental stimuli by directed growth and differentiation, and exhibit a range of senses. Generically, they are responsive to volatile and soluble chemicals, temperature gradients and physical parameters of the substrate, and may also respond to light, gravity and applied electric fields (Gooday, 1995). The most widely studied fungi are probably the pathogenic organisms, in which growth characteristics can often be related to disease incidence. Many tip growing cells are able to orient their direction of growth in relation to topographical changes in the substrate, an adaptation characteristic of organisms that grow through and live on solid surfaces and tissues which is likely to have an important impact on pathogenic invasion (Gow & Gooday, 1982; Sherwood *et al.*, 1992). *Candida albicans* Robin (Berkhout) Strain 3153 is an opportunistic pathogen of humans and is intimately associated with the epithelia. Work using artificial membranes with pores has suggested that contact sensing, or thigmotropism, is a contributive factor in epithelial invasion (Sherwood *et al.*, 1992), conferring similar penetrative advantages to those observed in plant pathogens which enter via the stomata. Zoosporic plant pathogens, particularly *Pythium* and *Phytophthora*, show homing responses mediated by recognition of chemical diffusates and surface components of the host and substrate (Deacon & Donaldson, 1993).

Hutchinson *et al.* (1980) studied factors controlling the development of *Mucor hiemalis* Wehm. Strain 2123 and concluded that the circular shape characteristic of fungal colonies growing from a single point on a plane, uniform surface was the expected result of any system with the branching and elongation patterns they observed, and that no other controlling factor was necessary. No evidence was found that orientating interactions between hyphal tips had any biological impact on the colony shape. The study of growth forms can have important commercial implications. Filamentous bacteria and fungi are important in biotechnology in, for example, the production of antibiotics. Morphology has an important role in the fermentation process. Optimum control of the process requires

knowledge of morphological parameters such as growth and branching rates, which are possibly correlated with productivity (Reichl *et al.*, 1990).

For maximum efficiency, hyphal growth must proceed in two or three dimensions and this can be achieved through branching to cover large areas and to communicate and translocate between regions. In general, branching frequency of fungal hyphae increases in areas of high nutrient concentration and is reflected to a large extent in fungal morphology (Prosser, 1983). It is recognised that both mechanisms of direction and of branching frequency contribute significantly to the efficiency with which fungi colonise solid substrata. Less is known about the mechanisms which determine the spatial distribution of the hyphae within the mycelial network (Trinci, 1984).

1.5 Arbuscular Mycorrhizal Hyphae

1.5.1 Role of AM Hyphae

AMF are known to vary in their capacity to produce external hyphae independent of their capacity to colonise the root cortex (Graham *et al.*, 1982), and although it is recognised that many chemical, physical and biological properties of soil that influence plant response to AMF act directly on the external phase, little is known about the specific effects of such factors on the distribution of these extramatrical hyphae (Sylvia, 1992). The distribution of the external hyphae is probably as important as their length in nutrient uptake (Abbott & Robson, 1985), but together with mechanisms by which spatial patterning may be achieved has seldom been evaluated (Friese & Allen, 1987). Many natural systems are not homogeneous on spatial scales that are relevant to the processes which occur within them. The potential ability of fungi to short-circuit physical and chemical constraints imposed on nutrient availability makes it important to understand the mechanisms of nutrient acquisition and distribution, since hyphal distribution is likely to influence both the acquisition of nutrients and the extent to which they are re-allocated within a medium. A functional understanding of structure, including the influence of resource distribution on morphology, is thus important (Crawford *et al.*, 1993).

Whether hyphae are distributed at random or selectively concentrated at nutrient rich microsites as a result of a specific locational mechanism will be reflected in nutrient acquisition (St. John *et al.*, 1983 a & b). Nutritional influences, such as acquisition of phosphorus beyond the root depletion zone, will have concomitant effects on plant growth enhancement (Graham *et al.*, 1982). If the nutrient status of a substrate is poor, a fungal colony will generally form low dimensional morphology and thus distribute as little hyphal mass as possible across a maximum area.

Conversely, on encountering a nutrient-rich site the hyphal dimension will increase and thus fill space with maximum efficiency for exploitation of the new substrate. For mycorrhizal fungi, it is currently considered that hyphae are unable to respond specifically to environmental cues in this way, and that random growth ultimately results in location of nutrient rich microsites (St. John *et al.*, 1983b; St. John & Coleman, 1983). The influence of resource distribution on morphology is thus important and reflects the ability of fungi to redistribute a patchily distributed nutrient source in the soil (Crawford *et al.*, 1993).

As it seems likely that mycorrhizal mycelia play a much wider role than previously attributed to them, there is an undoubted requirement for "real world", non-destructive measurements (Read, 1987) aimed at increasing understanding of processes in the soil through the simulation of conditions which more closely resemble natural systems. It has been demonstrated that the rate and pattern of spread of external hyphae from roots colonised by AMF varies greatly with the fungal isolate used, and it is likely that spatial distribution of individual species is important in terms of their ability to extend beyond the depletion zone and thus access greater amounts of soil phosphorus (Jakobsen *et al.*, 1992). Hyphal development parameters which place physical limitations on phosphorus fluxes could thus play a significant role in nutrient acquisition, but the distance that hyphae extend from roots, or their density in the soil, is seldom evaluated (Friese & Allen, 1987). Although it is recognised that mycorrhizal hyphae enable plants to obtain nutrients far beyond any rhizosphere depletion zone, their role in both phosphorus uptake and soil stabilisation is likely to be dependent on their distribution within the soil matrix relative to the root surface (Abbott *et al.*, 1992). It is thus hyphal distribution beyond this zone which is crucial if mycorrhizas are to be effective in nutrient uptake (Sanders & Tinker, 1971) and which will be as important as hyphal length in determining species effectiveness (Abbot & Robson, 1985). The question remains as to the mechanism by which spatial patterning is achieved and information is thus required on nutrient acquisition by hyphae within microsites (Friese & Allen, 1987). While it may seem reasonable to assume that fungal growth within and outwith the root are proportional, structural, functional and environmental differences are also implicated and species of AMF differ in the amount of external hyphae produced per unit length of colonised root (Schüepp *et al.*, 1987). The relationship between external hyphae and internal infection may be affected both by the properties of the soil and by the host plant phosphorus status (Abbot & Robson, 1985). It has been suggested that maximum development of external mycelium may occur only after maximum internal colonisation has been achieved (Sanders *et al.*, 1977), and that external mycelial growth may be controlled by factors

different to or independent of those that control colonisation (Graham *et al.*, 1982). Assessments of development should therefore ideally quantify extra-radical hyphae (Ames, 1987), and establishment of mycorrhizosphere boundaries is increasingly becoming recognised as an important research area.

External hyphae thus act as nutrient absorbing organs within a heterogeneous environment which contains a range of chemical and biological conditions at localised points. Within the soil volumes exploited by these hyphae some microsites are capable of yielding proportionately large amounts of nutrients, while others are of essentially no value in terms of nutrient supply. From observations in field and pot cultures it appears that extramatrical mycelium of AMF associate preferentially with particles of decomposing organic matter in the soil (eg. Mosse, 1959; Nicolson & Johnston, 1979). Similarly, mycorrhizal spores have been observed within other spores devoid of their original contents and it may be that these are a functional equivalent of organic debris, contributing space, nutrients and protection from degradative agents (Koske, 1984). It has been postulated that mycorrhizas may be capable of a limited saprophtytic existence (Nicolson, 1959; Warner & Mosse, 1980; Hepper & Warner, 1983). Alternatively, organic matter may simply provide a physiological cue for mycelial growth (St. John *et al.*, 1983a). Further evidence is, however, still required.

Mechanisms relating to hyphal distribution have generally been inferred from indirect evidence, as few studies have been carried out non-destructively. More recently, the adoption of two-dimensional systems has allowed direct observation of hyphae *in situ*, and has been used in experiments examining spore germination and early hyphal growth in response to root exudates and plant flavonoids (Gianinazzi-Pearson *et al.*, 1989). Visualisation of hyphae is improved in such systems by the use of direct image analysis techniques, or of image analysis of photographic enlargements (Hitchcock *et al.*, in press). However, the use of standard gel-based media in two-dimensional systems can result in problems due to contamination by other microbial species, and necessitate the use of axenic culture which is difficult to achieve in mycorrhizal systems.

1.5.2 AM Hyphal Morphology

Mycorrhizal hyphae have distinctive forms and dimensions (Read, 1984) and while little is known about many of their characteristics, basic morphological features have been recognised. The hyphae in the external phase of AMF are dimorphic, existing as thick-walled, aseptate hyphae which form the permanent basis of the external mycelial complexes in mature mycelial systems. From these branch thin-walled, often septate, hyphae which are generally temporary. These variations in wall

thickness are accompanied by variations in hyphal diameter. It has been suggested that this dimorphism reflects two functional distinctions within the absorptive mycelium: the viable hyphae, which are ephemeral hyphae of less than 5μm diameter able to both absorb and translocate nutrients, and the larger "trunk" hyphae which are non-viable and likely only to translocate nutrients, acting solely as a nutrient "pump" (Schubert *et al.*, 1987).

Until recently the inherent growth pattern of the AM endophytes received little consideration. Compared with that of common saprophytic and root pathogenic fungi the external soil-based mycelium of AM fungi appears to be relatively sparse, even under optimum conditions (Mosse, 1988). Friese & Allen (1991) identified two major hyphal forms growing out into the soil from the root surface. The runner hyphae are both structurally and functionally simple, consisting of long, single hyphal strands with angular projections, uneven wall thickness and few branches. These hyphae are apparently able either to track along the outside of the root of origin to produce multiple secondary infections, or to grow out into the soil matrix to infect other roots. They appear to have the principle function of seeking out new infectable roots. They are also able to form hyphal bridges between adjacent roots of the same, or different, plants. The absorptive hyphal network consists of a series of dichotomous branching orders which develop into a fan shaped network. Each successive order of hyphal branching is accompanied by a decrease in hyphal diameter, a pattern which allows an increase in the surface area : volume ratio of each consecutive hyphal cylinder. Individual hyphal filaments within a particular architectural type appear morphologically quite similar, and the complexity of the external hyphal system is based primarily on the unique function of each hyphal architecture (Friese & Allen, 1991). Diverse architectural forms are involved in this process since infection hyphae may originate from either spores or from root fragments (Friese & Allen, 1991).

Hyphae thus exhibit distinct architectural and functional characteristics. The runner hyphae are linear and unbranched and thus effective in finding new root fragments. They are the hyphae most able to initiate infection regardless of plant species. In contrast, the absorptive hyphal networks are efficient at exploiting the soil matrix resources, but not as units of infection. It is the mutualism inherent in the mycorrhizal symbiosis that allows the fungal hyphae to become so specialised in their structure and function (Friese & Allen, 1991). The overall architecture of the external hyphae may be limited by the rate of phosphorus inflow and carbon transport from the root to the mycelium and there is a continuous process of network formation and dieback (Friese & Allen, 1991). Whether or not different forms or proportions of each hyphal architecture are preferentially produced is not yet known. Longevity also

remains a little understood hyphal characteristic, for which few predictions have been made. Turnover may be very rapid, but development of hyphae may alternatively occur predominantly during the exponential phase of colonisation (Abbot & Robson, 1985).

1.6 Aims

The processes by which arbuscular mycorrhizal (AM) fungi colonise the plant root are probably among the most widely studied aspects of the symbiosis, at morphological, physiological and molecular level. Previous work has included studies of the responses of germ tube hyphae to external plant-related factors. In addition, the impacts of AM colonisation on plant growth and development have been widely recognised and researched.

The plant-soil link provided by the AM fungal hyphae is also an important aspect of the symbiosis, and is instrumental in the beneficial effects of the association to the plant. However, although the role of the extra-radical (ER) hyphae, particularly in nutrient acquisition, has been acknowledged, there have been relatively few attempts to study hyphal growth and morphology in detail, or to explore further observed hyphal responses to their environment. Notable previous work has shown that ER hyphae of AM fungi are responsive to heterogeneity of their growth medium, and can respond to organic particles in the soil by a change in branching rate (St. John *et al.*, 1983a & b). Observation of hyphal growth and development has also identified specialisation in both form and function in the ER hyphae (Friese & Allen, 1991).

This project aimed to develop a system in which ER hyphae of AM fungi could be grown *in vitro* for detailed study of their growth and morphology. Development of this system aimed to allow the removal of normal environmental influences on the hyphae, thus enabling specific substances to be tested under controlled conditions, and factors which influence hyphal growth and organisation within the mycelium to be identified. In this study, plant related factors were applied to AM fungal hyphae with the aims firstly of observing hyphal responses and explaining these in the context of the hyphal form and function, and secondly of identifying and discussing differences between germ tube and ER hyphal responses to similar environmental factors. The work also aimed to make basic identifications of bioactive compounds.

Chapter 2

2 Arbuscular Mycorrhizal Fungi *in vitro*

Collection of extra-radical mycelium and mycorrhizal roots from plants grown in soil systems cannot be carried out non-destructively. *In vitro* culture of arbuscular mycorrhizal fungi (AMF) to produce fungal material for research purposes, taxonomy and inoculation has therefore been frequently attempted though with variable success. The problems faced have restricted the progress of aseptic work, which could provide a potential starting point for pure cultures of AMF (Hepper, 1981). The culture of AMF in the absence of the host plant requires at least a simplification and standardisation of procedures. The factors on which the obligate relationship depends have not yet been elucidated, but there are many possible reasons why AMF cannot grow extensively and continuously unless part of a symbiotic partnership with a host root. These may be simple but essential nutrient requirements, or a more complex molecular need for the interaction with the host metabolism due to loss of genetic material by the AMF (Hepper, 1984). Axenically derived pure cultures of AMF may lose the ability to invade a root in the normal way, and it may thus be necessary to find a mechanism by which a change from saprophytic to symbiotic mode can be induced afer a period in culture (Hepper, 1984). Although it is possible to initiate a mycorrhizal symbiosis using a single spore as inoculum (eg. Hepper, 1981), axenic culture is ultimately dependent on the ability to subculture, which requires independent hyphal growth. As hyphal growth without attachment either to spore or root remains limited (Mosse, 1988), so does the ability to achieve this fundamental goal. Pure culture of AMF may remain unattainable because of disruption of an essential metabolic or biochemical process which requires a direct interaction between the fungus and host cells, thus preventing growth outside the symbiosis (Hepper, 1984).

Despite such problems as these, this type of work remains of potential use in negating the influence of other soil microbes in studies both of the biochemical aspects of fungal growth (Macdonald, 1981), and in nutrition and infection studies (Mosse & Phillips, 1971; Pearson & Tinker, 1975) to examine colonisation-induced changes in the host which are not attributable to the presence of other micro-organisms (Hepper, 1981). It also opens up the possibility of applying pure culturing techniques to the large scale production of AMF inoculum for field use (Hepper, 1984).

2.1 Source of Inoculum

In many studies spores have been preferred as the starting material, as they can be extracted relatively easily from soil by wet sieving, are capable of a short period of

independent growth after germination and offer advantages in terms of identification and relative ease of sterilisation (Mosse, 1988). Rapid spore germination can be achieved on simple media. Spores of *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerdemann and some other AMF have been shown to germinate readily on nutrient-free media although peptone (Macdonald, 1981) and phosphorus (Pons & Gianinazzi-Pearson, 1985) can promote hyphal growth. No or low phosphorus media have been observed to stimulate rapid and highly branched hyphal growth and to sustain this for approximately one month, resulting in the development of a sparse mycelium extending upto 16 mm from the spore (Pons & Gianinazzi-Pearson, 1985). The use of cellophane membranes have been observed to decrease hyphal growth from spores in some cases (Macdonald, 1981). However, the hyphae are restricted to a 2-D growth form which provides a greater inoculum potential for infection of axenic plants, due to an increased chance of an encounter between roots and hyphae. Alternatively, membranes can be used to separate the two components of the symbiosis in order to prevent contact and subsequent colonisation. Bécard & Piché (1989b) used dialysis membrane in this way to study hyphal elongation from spores in the presence or absence of roots, and in the presence of roots not in physical contact with the spores.

Results to date indicate the compromise between plant and fungal requirements in culture. Rapid plant cell growth requires high levels of both organic and inorganic compounds that are likely to cause inhibition of AMF spore germination and disruption of hyphal growth (Carr *et al.*, 1985). The combined culture of plant cells and AMF therefore requires modification of plant cell culture media. Carr *et al.* (1985) cultured plant cell suspensions using one-tenth strength Murashige & Skoog medium (Murashige & Skoog, 1962) with two grams per litre of sucrose to test interactions with surface sterilised spores of *Glomus caledonium* and *G. mosseae*. They concluded that while it was not possible to optimise all the components of the test medium, marked improvements in hyphal growth could be obtained from washed cell suspension cultures of wheat, lucerne and potato embedded in or added to the medium. The cell cultures were not differentiated as roots, or specifically derived from roots, indicating that stimulatory effects were not specific to a single plant species or cell type. Carr *et al.* (1985) concluded that volatile compounds released by the plant cells were responsible for the observed growth effects, and suggested the potential use of the method in pure culture of AMF. However, plant cell growth was not sustainable, limiting the use of cell suspensions in long term culture.

Due to the ability of spores to undergo multiple germinations, they may show a variability in germination time and hyphal growth potential that reflects the number of previous germinations and is thus not necessarily related to spore size (Mosse,

1988; Bécard & Piché, 1989b). An alternative source of mycorrhizal inocula can be provided by the use of infected root pieces. Regeneration of hyphae can occur from the cut ends of mycorrhizal root pieces placed in water or agar, particularly those containing intra-radical vesicles which are likely to increase inoculum infectivity (Biermann & Linderman, 1983; Hepper, 1984; Mosse, 1988). Use of mycorrhizal root pieces rather than spores may increase the risk of pathogen introduction (Ames & Linderman, 1978), but it has been acknowledged that the ability to disinfect and work with AMF taken directly from a soil based system would be an important step towards axenic culture (Williams, 1990).

Previous work with the obligately biotrophic rust fungi suggests that surface sterilised tissues containing intercellular mycelia provide more effective inocula for axenic culture than do spores (Williams, 1984). Extramatrical hyphae and intra-radical vesicles can be easily identified microscopically in unstained roots colonised by AMF, and a number of methods for surface sterilisation of mycorrhizal root pieces have been described. Mosse (1988) described a method of root piece sterilisation which achieved vigorous hyphal growth from 80% of root pieces with a growth rate of 0.3-0.5 mm per day. The sterilants used were 2% chloramine-T and 200 ppm streptomycin sulphate, and the root pieces were subsequently rinsed in sterile tap water. Regeneration occurred most freely from transversely cut root pieces of 1-1.5 mm in length, which contained many vesicles. Root pieces were incubated at 28°C on tap water agar, or on one-fifth strength G-T medium (Goforth & Torrey, 1977) with 0.8% Bacto agar.

For some species of AMF, intra-radical vesicles have been shown to increase the inoculum potential of excised mycorrhizae. *Glomus fasciculatum*, *G. mosseae* and *Acaulospora laevis* Gerdemann & Trappe, all mycorrhizal species which form intra-radical vesicles, showed increased root piece infectivity compared with species not generally forming vesicles. Separated vesicles retain infective potential and contribute significantly to root infectivity, potentially reducing the risk of contamination compared with root piece inoculum (Biermann & Linderman, 1983). That the ability to form intra-radical vesicles is important was highlighted in the work of Biermann & Linderman (1983), who found that root pieces colonised by *Gigaspora margarita* Becker & Hall were unable to infect geranium plants under conditions identical to those in which root inoculum of *Glomus mosseae*, *G. deserticola* Trappe, Bloss & Menge, *G. fasciculatum* and *Acaulospora spinosa* Walker & Trappe was effective. This was attributed to the absence of vesicles in *Gigaspora* colonised roots.

Techniques for total surface sterilisation of non-spore inoculum are largely unsuccessful. Sodium hypochlorite (0.5%), which is routinely used in sterilisation of

plant material for *in vitro* culture, has been found to kill AMF when used for ninety seconds and to decrease their infectivity after only thirty seconds. Below this, fungal pathogens are not eradicated (Biermann & Lindermann, 1983). Over a five year period, Williams (1990) developed a method of root piece sterilisation using decontamination by infiltration with sterile distilled water under vacuum, followed by immersion in 2% household bleach (nominally 4% available chlorine). The method produced an average of 22% (range 4-64%) uncontaminated root pieces in an incubation medium containing penicillin, streptomycin and bovine albumin, but these were able to form regrowth hyphae of only a few hundred micrometers (Williams, 1990).

2.2 Axenic Plant-AMF Systems

Attempts to produce mycorrhizal associations *in vitro* go back many years, but as yet AMF have not been sub-culturable on synthetic media without the presence of a host plant. Mosse (1962) described the initiation of new infections from mycorrhizal spores in the absence of other microbial populations, but found that colonisation was improved by the incorporation of pseudomonads or products of their culture filtrate. Successful growth of mycorrhizal plantlets has since been achieved under axenic conditions using numerous techniques, and microbial additions have been found to be unnecessary in nutritionally more balanced media (Mosse & Phillips, 1971). Hepper (1984) grew *Trifolium parviflorum* L. and *T. repens* L. plantlets colonised by *Glomus caledonium* and *G. mosseae* on agar slopes with soluble phosphorus or bone meal. Initial interaction between the fungus and plant root was characterised by a burst of hyphal growth corresponding to appressorium formation, or hyphal penetration between root cells before development of an intra-cellular (arbuscular) stage. Similar observations have been made in root organ cultures in which close contact between fungus and root surface is sufficient to produce hyphal growth stimulation (Mosse & Hepper, 1975). Colonisation of new seedlings placed on the agar slopes after removal of the colonised plant implicated the external hyphae in the infection process (Hepper, 1984). In addition to the agar slopes, Hepper (1984) also grew mycorrhizal plantlets on chromatography papers supported on microscope slides in test tubes, and on modified Fåhraeus slides in which roots were placed between a slide and cover slip at a fixed distance of 800μm. All the systems facilitated the initiation of a symbiosis using single spore inoculum. As previously observed in root organ cultures by Mosse & Hepper (1975), the infection site was often not at the point closest to the inoculum.

Mosse & Phillips (1971) were able to produce typical AM infections in axenic plants of the genus *Trifolium* without the need to pre-germinate the fungal spores. The development of infection was not dependent on the form in which phosphorus was supplied, but this did influence the relationship between internal and external development. Organic phosphate (calcium phytate and DNA) apparently stimulated extra-radical fungal growth. This effect may have been due to the inositol content, which is likely to have provided an additional source of carbon. However, in the presence of calcium phytate, the development of the fungus outside the root was found to be closely linked to that within. In contrast, where inositol itself was added to the medium, extra-radical development showed less dependence on internal colonisation, probably due to a further increase in fungal carbon supply over that supplied via the plant (Mosse & Phillips, 1971).

Due to the symbiotic nature of the plant:AMF relationship, hyphal elongation is retarded in the absence of roots. Growth can be significantly increased by the introduction of roots after spore germination, even without actual physical contact (Mosse & Hepper, 1975; Bécard & Piché, 1989b). Attempts to grow AMF in root organ cultures arose from a number of questions concerning the possibility of root infection by AMF in the absence of an attached shoot, and the potential of root organ cultures to provide an intermediate stage in the progression to pure culture (Hepper & Mosse, 1980). As new growth occurs from cut ends of intercellular hyphae, roots produced axenically which are infected with AMF potentially provide the starting material for culturing the fungi (Hepper, 1981), with the subsequent possibility of refining the system to produce axenic material in liquid culture as a basis for biochemical studies (Hepper & Mosse, 1980). Apparently typical infections, closely resembling those in a whole plant, were obtained in root cultures of *Trifolium pratense* L. even after these had been maintained by subculture for up to three years. Residual shoot material is thus unlikely to be playing a role in mycorrhizal initiation or development after a prolonged period *in vitro*, indicating that providing normal root growth can be maintained through an adequate carbon supply, AMF do not require metabolites specifically synthesised in the leaves (Mosse & Hepper, 1975; Hepper & Mosse, 1980). Mechanisms restricting the AMF to the primary cortex and preventing uncontrolled spread in the root also function independently of the leaves (Mosse & Hepper, 1975). However, infections failed to keep pace with root growth when the colonised roots were subcultured, and the possibility that root organ cultures could provide an intermediate stage in the axenic culture of AMF remains unfounded.

Although Mosse & Hepper (1975) did achieve successful and apparently typical root colonisation by AMF in culture, only 53 out of 163 cultures became

mycorrhizal. The use of root organ cultures under axenic conditions does, however, allow the initiation of the symbiosis via either spores or mycorrhizal root segments, and enables non-destructive observations of fungal development in an artificial environment. The use of carrot roots transformed by *Agrobacterium rhizogenes* Strain A4 provides a defined and reproducible system for standardised quantitative measurements of fungal growth under these conditions (Bécard & Fortin, 1988; Bécard & Piché, 1989b).

Successful infection in root cultures depends largely on the condition and proximity of nearby roots rather than on the number or size of spores in the inoculum, although freshly germinated spores are preferable. There does not appear to be any attraction between germ tubes and roots unless they are in very close proximity, but contact between rapidly extending germ tubes and young lateral roots will result in extensive infection (Mosse & Hepper, 1975). Such infections, apparently identical to those found in whole plants, are followed by vigorous growth of external mycelium extending 30-40 mm from the root surface (Mosse & Hepper, 1975).

Inoculation does not necessarily lead to infection, and in some instances vigorous hyphal growth can occur without any evidence of root penetration. Subculture from external mycelium remains unsuccessful, and *in situ* growth is not sustainable following removal of an infected root (Mosse & Hepper, 1975). Attempts to use root or shoot callus as a route to axenic culture by infection with AMF also remain unsuccessful (Hepper & Mosse, 1980).

This part of the study aimed to exploit current knowledge of AMF culture systems to develop a sterile system suitable for *in vitro* growth and observation of the extra-radical hyphae of AMF. Successful development of a culture system was necessary to provide a basis for further work on more detailed aspects of hyphal responses to applied test substances.

The use of extra-radical hyphae as the biological material for the study raised immediate difficulties concerning inoculum sterility. The need to separate hyphae and host plant to allow the study of specific plant-related compounds on hyphal growth and development caused further difficulties in terms of both culturing and sterility. This compromise between hyphal growth and sterility ultimately raised important questions which directed subsequent work. These questions are discussed in sections 2.6 and 3.9.

2.3 Methods

2.3.1 Sterilisation Techniques

Root piece sterilisation experiments were carried out in an attempt to produce sterile mycorrhizal inoculum for growth experiments. The methods used for mycorrhizal inoculation of stock plants and for collection of mycorrhizal root piece inoculum are described in detail in Chapter 3 (Sections 3.3.1 and 3.3.3).

2.3.1.1

1mm mycorrhizal root pieces were sterilised in chlorine-based household bleach (Domestos, Lever Ltd., UK; available chlorine <5%) which is routinely used in plant cell and tissue culture techniques and has also been reported for use in sterilisation of mycorrhizal root pieces (Williams, 1990). The root pieces were placed into a small 50µm mesh (Plastok Associates Ltd., UK) filter made from a modified syringe barrel, and allowed to stand in 5%, 10% or 15% (v/v) Domestos for durations of 5, 10 or 15 minutes. 8 replicates were used for each treatment. After sterilisation the root pieces were rinsed thoroughly in sterile tap water and transferred aseptically to disposable multi-well plates (Cell Wells™, Corning Scientific Products, USA) containing 250µl sterile nutrient broth (Oxoid, UK) per cell. Nutrient broth is a standard growth medium for bacteria, which becomes cloudy when contaminated. The plates were incubated in the dark at 28°C for 6 days and checked regularly, both for contamination and for hyphal regrowth, using a binocular microscope. The proportion of pieces showing bacterial contamination was calculated for each treatment (Table 2.1; Section 2.4.1.1).

The most effective treatment (15% v/v Domestos for 10 minutes: Table 2.1; Section 2.4.1.1), in which no contamination occurred within the 6 day incubation period, was repeated (n=84). After incubation the root pieces were plated out onto potato dextrose agar (PDA; Oxoid, UK) which is a standard fungal growth medium, and observed microscopically for hyphal regrowth.

2.3.1.2

As a result of data obtained from the experiments above (reported in section 2.4.1.1), lower concentrations of Domestos were applied for shorter time periods using the same methodolgy. In this experiment, 5% and 10% (v/v) Domestos were used for 5 minutes. In addition, chloramine-T (N-chloro-p-toluene-sulfonamide sodium salt; Sigma Chemical Co., USA), a sterilant routinely used in spore sterilisation procedures, was tested on root pieces at a concentration of 5% (w/v) for

a duration of 10 minutes. The sterilised root pieces were transferred aseptically onto disposable Petri dishes (90 mm, triple vent; Bibby Sterilin Ltd., UK) containing PDA (Oxoid, UK), and incubated at 28°C in the dark. The dishes were checked to determine the percentage contamination (Table 2.2; Section 2.4.1.2), and microscopic examination for hyphal regrowth was carried out using a binocular microscope (Wild M10, Leica, UK).

2.3.1.3

Further sterility tests using Domestos at the range of concentrations described in (2.3.1.1), but for a reduced time period (5%, 10% & 15% for 5 minutes) were carried out in an attempt to achieve hyphal regrowth after sterilisation. In this experiment, sterilised root pieces were incubated at 28°C for 7 days between moistened sterile cellulose nitrate filters (45µm pore size, 47mm diameter; Whatman Paper Ltd., UK) and tested for hyphal regrowth by destructive cold staining with trypan blue for 5 minutes. Microscopic observation was carried out to assess hyphal development.

2.3.1.4

The choice of sterilant was amended to 5% chloramine-T (Sigma Chemical Co., USA) in combination with 0.05% streptomycin sulphate (w/v) (Sigma Chemical Co., USA) (modified methods of Gianinazzi-Pearson *et al.*, 1989 and Williams, 1990) with a few drops of Tween 80 (polyoxyethylene (20); BDH Laboratory Supplies, UK) as a surfactant. These were made up in sterile water and filter sterilised through 0.2µm syringe filters (Nalgene Brand Products, Sybron Corporation, USA). Root pieces were sterilised for 10 minutes, rinsed thoroughly in sterile tap water and transferred aseptically to the incubation medium in disposable Petri dishes (Bibby Sterilin Ltd., UK). These were incubated at 28°C in the dark and examined regularly under a binocular microscope for hyphal regrowth.

In this experiment, a number of incubation media were tested to assess their influence on the regrowth capacity of the mycorrhizal hyphae:

i) cellulose nitrate filter (Whatman Paper Ltd., UK) sandwich with water storing granules (SwellGel, Glowcroft Ltd., UK) to retain moisture
ii) single cellulose nitrate filter with water storing granules to retain moisture
iii) cellulose nitrate filter sandwich with damp cotton wool pads to retain moisture

iv) single cellulose nitrate filter with damp cotton wool pads to retain moisture

v) Phytagel (Sigma Chemical Co., USA); 1 g l^{-1} + 0.05% Streptomycin sulphate (Sigma Chemical Co., USA)

vi) Phytagel (1.5 g l^{-1}) + 0.05% Streptomycin sulphate in a divided Petri dish (Bibby Sterilin Ltd., UK), with either damp cotton wool or water storing granules in the second half of the dish to retain moisture

vii) Transfergel (Sigma Chemical Co., USA); 0.2 g l^{-1} + 0.05% Streptomycin sulphate

2.3.1.5

Following results from previous experiments, the sterilant used was amended to 2% chloramine-T (Sigma Chemical Co., USA) in combination with 0.02% Streptomycin sulphate (Sigma Chemical Co., USA) (modified method of Mosse, 1988). The sterilants were made up in water and filter sterilised through 0.2µm syringe filters (Nalgene Brand Products, Sybron Corporation, USA). Root pieces colonised with *Glomus tenue* (Greenhall) Hall, *Glomus E3*, *Glomus etunicatum* Becker & Gerdemann or *Glomus intraradices* were sterilised for 10 minutes, rinsed thoroughly in sterile tap water and transferred aseptically onto sterile cellulose nitrate filters (Whatman Paper Ltd., UK) in disposable Petri dishes (Bibby Sterilin Ltd., UK). The root pieces were covered with a second filter and kept moist between damp cotton wool pads. The plates were sealed with Nesco film (Bando Chemical Industries Ltd., Japan) and incubated at 28°C in the dark. After 7 days, the filters were cold stained for 5 minutes with trypan blue, and hyphal growth was examined using a binocular microscope (Table 2.3; Section 2.4.1.5).

High power microscopy (Polyvar Fluorescence Microscope, Reichert-Jung, Austria) was used for more detailed examination of stained root pieces after mounting sections of the cellulose nitrate filters in polyvinyl lactophenol, and covering with glass coverslips.

2.3.1.6

Due to problems with contaminants in the microcosms (Section 2.3.2), originating from root pieces used as mycorrhizal inoculum, the sterilant was amended by the addition of 10 µg l^{-1} tetracycline hydrochloride (Sigma Cemical Co., USA). This antibiotic was chosen as a result of tests in which bacterial contaminants were isolated and plated out in the presence of antibiotic impregnated discs (M. Munro, pers. comm.).

Root pieces colonised by *Glomus etunicatum* were rinsed with sterile water, and then shaken in the sterilant for 30 minutes on an orbital shaker (Edmund Bühler, Germany) at 200 rpm. The root pieces were then rinsed thoroughly in sterile tap water and transferred aseptically onto sterile cellulose nitrate filters (Whatman Paper Ltd., UK) in disposable Petri dishes (Bibby Sterilin Ltd., UK). The filters were covered with a second filter and kept moist between damp cotton wool pads. The plates were sealed with Nesco film (Bando Chemical Industries Ltd., Japan) and incubated at 28°C. After 7, 10 and 14 days replicate filters were cold stained for 5 minutes with trypan blue, and hyphal growth examined using a binocular microscope (Table 2.4; Section 2.4.1.6).

High power microscopy (JenaMed 2, Carl Zeiss Jena, Germany) was used for more detailed examination of stained root pieces after mounting sections of the cellulose nitrate filters in polyvinyl lactophenol.

The same sterilisation technique was subsequently repeated with a number of incubation media to assess the development of bacteria observed within the root pieces:

(i) Phytagel (Sigma Chemical Co., USA)
(ii) Phytagel + 0.05% streptomycin sulphate
(iii) Nutrient agar (Oxoid, UK)
(iv) Cellulose nitrate filters (as controls)

In subsequent microcosm experiments, 0.02% (w/v) streptomycin sulphate and 10µg l^{-1} tetracycline hydrochloride were included in the medium.

2.3.2 Design of Experimental Units

An experimental system in which mycorrhizal hyphae were restricted to a 2-dimensional growth pattern was developed using disposable Petri dishes (Bibby Sterilin Ltd., UK) as the basic experimental unit. Initially, circular (7cm diameter) dishes were used. These contained Phytagel (Sigma Chemical Co., USA) which was overlaid with dialysis membrane (molecular weight cut-off 12-14000; Medicell International Ltd., UK) to prevent hyphal growth into the medium (Allan, 1983; Bécard & Piché, 1989b). The membranes were boiled twice for 30 minutes each time in de-ionised water to remove plasticisers, and autoclaved between moist filter papers which prevented crinkling. They were then transferred aseptically onto the surface of the gel and inoculated with surface sterilised mycorrhizal root pieces. The plates were sealed with Nesco film (Bando Chemical Industries Ltd., Japan) and incubated at

28°C in the dark. Subsequently, this method was modified by the addition of a thin film of gel poured over the membrane, thus retaining moisture while maintaining the 2-dimensional growth pattern.

The system was later further modified to use square (10 x 10cm) disposable Petri dishes (Bibby Sterilin Ltd., UK) to which plantlets (Section 2.3.3) were also added. A slit was melted down through one edge of the dish, and through the equivalent edge of the lid, using a hot scalpel blade. Pre-germinated cucumber seedlings were placed into the experimental units so that the roots were within the dishes growing over the thin upper layer of gel, and the shoots were protruding through the slit from the dish to the external atmosphere. The stems were sealed into the slits using lanolin (BDH Laboratory Supplies, UK) to prevent contamination. Two techniques were initially used:

i) non-inoculated plantlet + sterile mycorrhizal root pieces
ii) pre-inoculated mycorrhizal plantlet (Section 2.3.3)

A third technique was then introduced in an attempt to improve visualisation of hyphae. In this method, the plantlets were placed over the main volume of the gel and the membrane and thin upper layer of gel were subsequently added over the top. Sterile mycorrhizal root pieces were then inoculated onto the upper layer of gel (Figure 2.1). This enabled separation of the root piece and the whole plant roots, while allowing diffusion of root-derived compounds that may be necessary for hyphal regrowth.

These mini-microcosms were foil-wrapped to exclude light from the plant root system, and maintained under light banks with a 16:8 hour light:dark regime. Hyphal regrowth was observed under sterile conditions using dark field illumination on a binocular microscope.

2.3.3 Plantlet Production

Sterile cucumber plantlets (cv's Burpless Tasty Green & Bush Champion, Hydra/Chase, The Organic Gardeners, UK) were produced by germination of seeds surface sterilised in 10% Domestos for 10 minutes, and rinsed thoroughly in sterile tap water. The seeds were placed between sterile cellulose nitrate filters (Whatman Paper Ltd., UK) and kept moist between damp cotton wool pads in Petri dishes (Bibby Sterilin Ltd., UK). Germinated seedlings were either tranferred directly into the mini-microcosms, or inoculated with surface sterilised mycorrhizal root pieces and

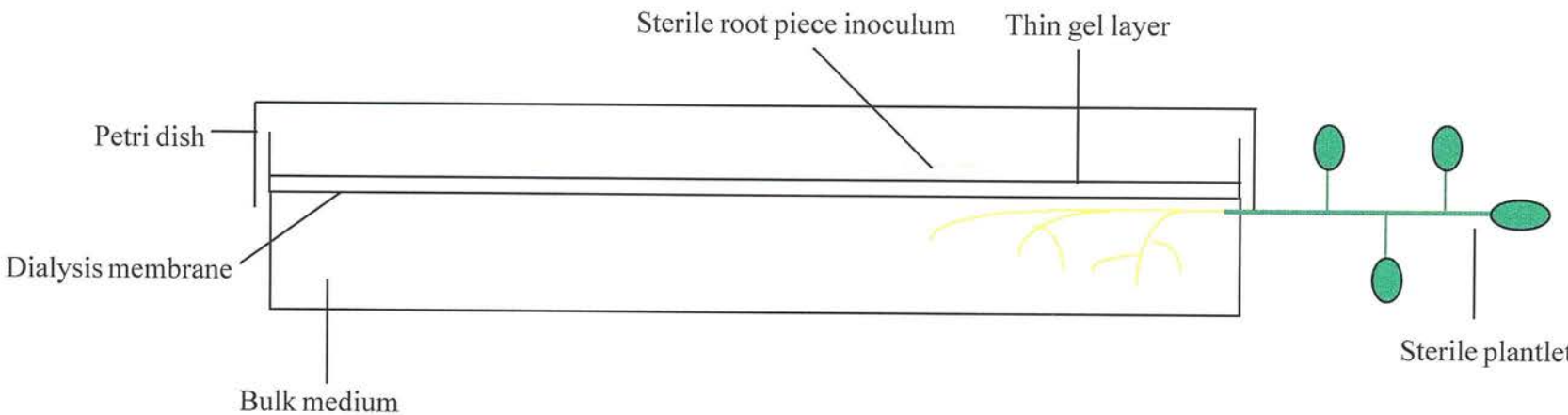


Figure 2.1: Microcosm developed for observing hyphal growth in the presence of a plant root system, with root piece inoculum separated by a membrane to prevent root contact

incubated for approximately 7 days to produce pre-mycorrhizal sterile plantlets for use in experiments.

2.4 Results

2.4.1 Sterilisation Techniques

2.4.1.1

Root piece sterilisation was carried out using 1mm transverse root sections, containing vesicles, harvested from stock plants colonised by the mycorrhizal fungus *Glomus E3*. Domestos (Lever Ltd., UK) was used as the sterilant at concentrations of 5%, 10% or 15% (v/v) for durations of 5, 10 or 15 minutes. Sterilised root pieces were incubated in nutrient broth at 28°C. Results, presented as percentage root pieces contaminated, showed that all treatments were able to prevent contamination for at least 1 day (Table 2.1). After more prolonged incubation, observation showed that sterilisation became more effective as the concentration of Domestos increased. Rapid increases in percentage contamination were observed in all treatments using 5% Domestos, irrespective of duration. Duration of treatment also apparently had little effect where 10% Domestos was used as the sterilant. Conversely, at the highest concentration (15%), effectiveness was increased with increased duration of treatment, although the most effective treatment was 15% for 10 minutes and not the maximum combination of 15% for 15 minutes (Table 2.1).

When the experiment was repeated using the most effective treatment (15% Domestos/10 minutes) only 4 of the 84 root pieces (4.76%) were contaminated after 8 days incubation at 28°C. However, clean root pieces transferred aseptically onto potato dextrose agar (PDA; Oxoid, UK) in disposable sterile Petri dishes (Bibby Sterilin Ltd., UK) and incubated at 28°C failed to germinate, even when subsequently transferred into dishes containing sterile plantlets.

2.4.1.2

Due to the apparent hyphal growth suppression observed in root pieces from tests carried out using more concentrated and prolonged treatments with Domestos, root piece sterilisation was repeated using 5% (v/v) and 10% (v/v) Domestos for only 5 minutes, and also 5% (w/v) chloramine-T (Sigma Chemical Co., USA) for 10 minutes. Root pieces were incubated on PDA at 28°C.

Root pieces treated with 5% and 10% Domestos for 5 minutes showed similar levels of contamination after 13 days (40.0 & 39.29% respectively; Table 2.2). Root pieces treated with chloramine-T showed much more rapid development of contamination, and all were contaminated by day 13 (Table 2.2). No hyphal regrowth was observed from any of the uncontaminated root pieces.

Table 2.1 Development of contamination (%) in mycorrhizal root pieces sterilised in Domestos and incubated at 28°C in multiwell plates containing 250µl nutrient broth.

Figures are means of 8 replicates

Time (d)	Domestos (%)								
	5			10			15		
	Duration (mins)								
	5	10	15	5	10	15	5	10	15
1	0	0	0	0	0	0	0	0	0
4	62.5	50.0	62.5	25.0	12.5	50.0	50.0	0	12.5
6	75.0	62.5	75.0	37.5	25.0	50.0	50.0	0	12.5

Table 2.2 Development of contamination (%) in sterilised mycorrhizal root pieces incubated at 28°C on potato dextrose agar.

Figures are means of 5, 28 and 26 replicates

Time (d)	Sterilant		
	Domestos (%)	Domestos (%)	Chloramine-T (%)
	5	10	5
	Duration (min)		
	5	5	10
2		14.29	65.38
3		32.14	73.08
7		39.29	96.15
13	40	39.29	100.00

2.4.1.3

Root pieces sterilised using 5%, 10% or 15% (v/v) Domestos for 5 minutes were incubated between sterile cellulose nitrate filters (Whatman Paper Ltd., UK) at 28°C, and assessed for hyphal regrowth by cold staining for 5 minutes with trypan blue. After 6 days, only root pieces sterilised in 5% Domestos were showing hyphal regrowth, indicating that higher concentrations of Domestos were retarding hyphal development. After further incubation, more replicates were stained and examined 11 days after sterilisation. Again, growth was most pronounced from root pieces sterilised in 5% Domestos, although a single root piece sterilised in 15% Domestos did show limited hyphal development. This result indicated that incubation media as well as the harshness of the sterilisation technique could be a contributory factor in hyphal regrowth capacity, as in previous experiments root pieces sterilised with 15% Domestos were not able to produce regrowth hyphae when incubated on PDA. It subsequently became apparent from the literature that standard fungal growth media such as PDA are inhibitory to mycorrhizal fungi (Gerdemann, 1955; Mosse, 1988).

2.4.1.4

A number of incubation media were tested using root pieces sterilised with 5% (w/v) chloramine-T + 0.05% (w/v) streptomycin sulphate (Sigma Chemical Co., USA) for 10 minutes. Hyphal regrowth did not occur under any of these experimental conditions, including the cellulose nitrate filter sandwich technique which provides conditions under which hyphae will generally grow from non-sterile root pieces. This suggested that the sterilant itself was preventing hyphal growth.

2.4.1.5

Root piece sterilisation was carried out using 2% (w/v) chloramine-T + 0.02% (w/v) streptomycin sulphate for 10 minutes. The sterilised root pieces were incubated at 28°C between cellulose nitrate filters (Whatman Paper Ltd., UK), and hyphal regrowth was assessed after 7 days by cold staining with trypan blue.

After 7 days, hyphae in root pieces colonised by both *Glomus tenue* and *Glomus etunicatum* had begun to regrow. Hyphal development from *Glomus tenue* was restricted to 25% of root pieces, and hyphal growth was not extensive. In contrast, extensive regrowth hyphae were present in 75% of root pieces colonised by *Glomus etunicatum*. Root pieces colonised by *Glomus E3* and *Glomus intraradices* did not produce regrowth hyphae, and those colonised with *Glomus E3* showed considerable fungal contamination. Non-sterile control root pieces all produced regrowth hyphae.

Replicates assessed after 14 days incubation showed slightly different results, with no development of mycorrhizal regrowth hyphae from *Glomus tenue*, *Glomus E3* or *Glomus intraradices*; *Glomus E3* was contaminated with non-mycorrhizal fungal species. *Glomus etunicatum* did exhibit hyphal regrowth.

Table 2.3 Presence (+) or absence (-) of regrowth hyphae from mycorrhizal root pieces sterilised with 2% chloramine-T + 0.02% streptomycin for 10 minutes, and incubated between cellulose nitrate filters at 28°C.

Time (d)	Mycorrhizal Species			
	Glomus tenue	*Glomus E3*	*Glomus etunicatum*	*Glomus intraradices*
7	+	-	+	-
8	-	+	+	-
14	-	-	+	-

High power microscopic observation (Polyvar Fluorescence Microscope, Reichert-Jung, Austria) of mounted sections of the stained cellulose nitrate filters showed the presence of rod shaped bacteria (probably a *Bacillus* species) both inside and outside the root pieces. The *Glomus E3* colonised root piece was also contaminated with a large, branched non-mycorrhizal hyphal network with clearly defined septa between which the stain was particularly concentrated. Regrowth hyphae from *Glomus etunicatum* colonised root pieces were also infected by the rod shaped *Bacillus* at the ends nearest to the root. *G. etunicatum* was the only species in which regrowth hyphae were routinely found after sterilisation (Table 2.3). This species and sterilisation technique were subsequently used in tandem for further experiments.

2.4.1.6

Sterilisation of transverse root sections colonised by *Glomus etunicatum* was carried out using 2% (w/v) chloramine-T, 0.02% (w/v) streptomycin sulphate and 10μg l^{-1} tetracycline hydrochloride (Sigma Chemical Co., USA) for 10, 30 or 60 minutes with continuous agitation at 200 rpm on an orbital shaker (Edmund Bühler, Germany). The sterilised root pieces were incubated at 28°C between cellulose nitrate filters (Whatman Paper Ltd., UK) and hyphal regrowth was assessed after cold staining with trypan blue.

After 7 days, hyphal regrowth was observed from root pieces in all treatments. Regrowth had occurred in 33.3% of those sterilised for 10 minutes (Table 2.4). A further 33.3% showed severe fungal contamination, suggesting that this procedure did

not remove contaminants. The remainder of the root pieces in this treatment were uncontaminated, but showed no development of mycorrhizal hyphae. In contrast, 83.3% of root pieces sterilised for both 30 and 60 minutes exhibited regrowth of mycorrhizal hyphae (Table 2.4). Hyphal development was more prolific in the 30 minute treatment. Root pieces sterilised for 30 and 60 minutes did not show immediate evidence of contamination. However, when observed using high power microscopy (JenaMed 2, Carl Zeiss Jena, Germany), bacteria (*Bacillus* species) were evident within the root pieces.

Table 2.4 Hyphal regrowth (%) from replicate mycorrhizal root pieces sterilised using 2% chloramine-T, 0.02% streptomycin sulphate & 10μg l^{-1} tetracycline hydrochloride for 10, 30 & 60 minutes, incubated at 28°C between cellulose nitrate filters and stained destructively with cold trypan blue.

Figures are means of 6, 8 and 4 replicates

Time	Duration (mins)		
(d)	10	30	60
7	33.3	83.3	83.3
10	75.0	75.0	50.0
14	75.0	75.0	66.0

After 10 days incubation, replicate root pieces sterilised for 60 minutes showed the lowest hyphal regrowth (50%), while 75% of those sterilised for 10 and 30 minutes had developed regrowth hyphae (Table 2.4). This trend was still apparent after 14 days, when 66% of root pieces sterilised for 60 minutes had regrowth hyphae, compared with 75% of those treated for 10 and 30 minutes (Table 2.4). However, 25% of the root pieces treated for only 10 minutes showed fungal contamination, again indicating the inadequacy of this treatment. Fungal contaminants were distinguished from mycorrhizal hyphae by their growth form, which consisted of hyphae radiating outwards from the point of contamination to form a circular colony.

The experiment was repeated using the 30 minute sterilisation period, and the root pieces were incubated on a number of media (nutrient agar; Phytagel ± 0.05% streptomycin sulphate; cellulose nitrate filters). These media were tested in an attempt to assess external bacterial proliferation from bacterial cells observed within the root pieces using high power microscopy. Bacterial growth was observed in 25% of the root pieces incubated on nutrient agar after only 24 hours (Table 2.5). After 2 days,

40% of root pieces showed development of bacterial colonies. No contamination was observed in root pieces incubated on Phytagel ± 0.05% streptomycin sulphate within this time period (Table 2.5).

After a 3 day incubation period there had been no further increase in contamination levels on the nutrient agar plates. However, bacterial contamination had developed in 30% of the root pieces incubated on phytagel. No contamination was evident in the root pieces incubated on phytagel + streptomycin sulphate (Table 2.5). After 6 days incubation, contamination was evident in only 5% of the root pieces incubated on phytagel + streptomycin sulphate, and bacterial growth was very limited compared to colony development on the nutrient agar and phytagel plates. In contrast, 45% and 40% of root pieces incubated on nutrient agar and phytagel respectively were severely contaminated (Table 2.5). These levels of contamination had not changed after 10 days incubation.

Table 2.5 Development of contamination (%) in mycorrhizal root pieces sterilised using 2% chloramine-T, 0.02% streptomycin sulphate & 10μg l^{-1} tetracycline hydrochloride for 30 minutes.

Figures are means of 20 replicates

Time (d)	Incubation Medium		
	Nutrient Agar	Phytagel	Phytagel + Streptomycin
1	25.0	0	0
2	40.0	0	0
3	40.0	30.0	0
6	45.0	40.0	5.0
7	45.0	40.0	5.0
10	45.0	40.0	5.0

2.4.2 Experimental Unit Design

The use of transparent gel (Phytagel, Sigma Chemical Co., USA) as the incubation medium in the microcosms allowed enhanced microscopic visualisation of living hyphae compared to systems using cellulose nitrate filters (Whatman Paper Ltd., UK) which required visualisation by destructive staining techniques. The first method attempted, which used a single layer of gel overlaid with dialysis membrane (Medicell International Ltd., UK) in a disposable circular Petri dish (Bibby Sterilin Ltd., UK), resulted in drying out of the root pieces because of an inadequate moisture

supply. The addition of a thin upper layer of gel overcame this problem, and when the methodology was transferred to 10x10cm square Petri dishes produced a successful experimental system. The use of the membrane and thin gel layer for separation of the plantlet and root pieces was adopted as the technique most likely to facilitate non-destructive hyphal visualisation. Mycorrhizal hyphae were visible using a binocular microscope with dark field illumination (Wild M10, Leica, UK).

Although all three methods of plantlet introduction were successful, the method of sterilisation using 2% (w/v) chloramine-T (Sigma Chemical Co., USA) + 0.02% (w/v) streptomycin sulphate (Sigma Chemical Co., USA) proved to be inadequate when applied in this experimental situation. After amendment of the sterilant by the addition of 10μg l^{-1} tetracycline hydrochloride, there was no evidence of contamination in the microcosms. Pre-infected mycorrhizal plantlets (Section 2.3.3) showed apparent abundance of extra-radical hyphae. These were visualised firstly using dark field binocular microscopy, and then by cold staining *in situ* with trypan blue. Photographs were taken through a macro-viewer onto disk via the Quantimet 600 image analysis system (Leica, UK).

2.5 Conclusion: Summary of Experimental Results

This section of the work concentrated on developing a method for surface sterilisation of mycorrhizal root pieces, with the aim of setting up a sterile culture system to facilitate observation of AM fungal hyphae both in the presence and absence of a plant.

A number of sterilisation techniques were tested, and results are summarised below:

Domestos (available chlorine <5%)

Increasing concentrations of Domestos up to 15%, and durations of treatment up to 10 minutes, improved root piece sterility. Lower concentrations and durations of treatment were not effective in decontamination of root pieces.

However, hyphal growth was compromised by this treatment.

Chloramine-T (C-T)

Sterilisation using 5% C-T for 10 minutes did not effectively decontaminate root pieces.

Chloramine-T (C-T) + Streptomycin Sulphate (SS)

Duration of treatment 10 minutes:

5% C-T + 0.05% SS No hyphal regrowth occurred from root piece inoculum.

2% C-T + 0.02% SS Hyphal regrowth occurred, but root piece decontamination was not effective.

2% C-T, 0.02% SS + 10µg l^{-1} Tetracycline Hydrochloride

10 minutes Hyphal regrowth occurred, but treatment was unable to completely remove fungal contaminants.

30 minutes Hyphal regrowth occurred. There was no immediate evidence of contamination but *Bacillus* species were found to be present.

60 minutes Hyphal regrowth occurred, but appeared to be suppressed by the treatment. There was no immediate evidence of contamination, but *Bacillus* species were found to be present.

Incubation media were also tested:

The use of a conventional fungal growth medium was suppressive to growth of AMF. The addition of 0.05% streptomycin sulphate to the medium prevented spread of contamination from root piece inoculum.

A system for non-destructive observation of AM fungal hyphae was developed using 10 x 10 cm square Petri dishes containing transparent gel, with dialysis membrane to separate the plant root system and the mycorrhizal root piece inoculum. This allowed observation of hyphae in the presence of the root system, while preventing their direct contact with it.

2.6 Discussion

In vitro systems have been routinely applied in plant regeneration and micropropagation for several decades (Wiemken, 1995), but their application in the long term and maintainable culture of AMF in the absence of the host plant has as yet proved unsuccessful. Interest in *in vitro* systems has been heightened by research interest in fundamental aspects of the plant-fungal interaction, but both the development and standardisation of such systems have proved difficult, largely because of the obligate nature of the symbiosis and the consequent inability to maintain and subculture AMF. Under natural conditions AMF require association with root cortical tissues for growth and development, and although spore germination and extensive germtube growth has been attained under laboratory conditions (Hepper, 1984), neither the subculture of AM fungal hyphae nor fungal differentiation and sporulation have been achieved. This lack of success is despite systematic screening of defined and complex media for AMF hyphal growth and differentiation, and as such presents a challenge central to mycorrhizal research. The problem inherent to resolving this challenge lies within the difficulty of the investigation and characterisation of the biology of an organism which cannot be grown in isolation, and in the difficulty of successfully developing a system for growing it in isolation when its fundamental biology is not fully understood (Wood & Cummings, 1992).

Sterilisation Techniques

Root piece sterilisation gives rise to a number of problems that are not encountered with the use of spores as the starting material in studies of AMF. These problems occur largely because of the difficulties associated with removing internal contaminants without adverse effects on the AMF itself. Lack of information concerning conditions for growth of AMF *in vitro* causes additional problems with respect to the incubation medium used. Attempts to define requirements for spore germination and germ tube growth on artificial media have had variable success, and difficulties have probably been aggravated by variation in methodologies, culture conditions and fungal species (Siqueira *et al.*, 1982). Application of such techniques to root piece inoculum has seldom been attempted, and is open to a greater degree of variability generated by the inoculum itself. The use of root pieces rather than spores as the source of inoculum was central to the primary aim of this study, which was to enable the generation of extra-radical hyphae of AMF, and thus to study growth effects on this phase of the symbiosis. This is discussed in detail in Section 3.9.

As with *in vitro* techniques in general, methods of sterilisation routinely applied in plant culture systems were found not to be directly transferrable to AMF. Thus, both the concentration and duration of treatment with Domestos (Lever Ltd., UK) were found to be important. Low concentrations of Domestos were unable to prevent contamination from occurring irrespective of the duration of treatment, indicating the difficulties of sterilising the interior of the root piece. Conversely, while higher concentrations of Domestos were successful in sterilising the root pieces, the regrowth potential of AMF hyphae was compromised. Chloramine-T, used previously in surface sterilisation of root pieces in combination with antibiotics (Mosse, 1988) was unsuccessful in this study when applied at a higher concentration. Similarly, hyphal growth was retarded.

Tests of growth media indicated the sensitivity of AMF to cultural conditions. Standard fungal growth media were observed to be detrimental to AMF hyphal growth. This problem was previously encountered by Mosse (1988) who found that most traditional tissue culture media were too nutrient-rich for establishment of mycorrhizal infection, and that standard fungal growth media such as potato dextrose or malt extract inhibited even limited growth from germinating spores. No attempt was made to explain why this should be the case, but it seems likely that differences in the method of nutrient acquisition by AMF associated with their symbiotic status affect their sensitivity to external sources of nutrients in comparison with other types of fungi. Macdonald (1981) used nutrient media to screen spores for contamination after surface sterilisation. Using spores it is possible to ascertain sterility prior to germination and the onset of hyphal growth, and thus hyphal growth is unlikely to be retarded. In contrast, AM fungal structures within root piece inoculum are vulnerable to exposure to the growth medium.

Using the sterilisation method described by Mosse (1988), hyphal regrowth from mycorrhizal root pieces was obtained on cellulose nitrate filters (Whatman Paper Ltd., UK) which provide an inert growth medium. Differences in hyphal development between the four species of AMF used indicated specific differences in response to sterilisation treatments. Individual fungi are already known to differ in their infectivity, as measured by time of commencement of infection, amounts of infection produced at a given inoculum density and strategies by which spread of infection occurs (Abbott & Robson, 1981; Wilson, 1984). It thus seems likely that extra-radical growth differences may also arise between species.

Because sterilisation procedures must be mild enough not to damage the AMF, they are rarely completely effective (Macdonald, 1981). However, the use of strong disinfectants is particularly inappropriate for sterilisation of AMF in delicate

root tissues (Williams, 1990). In this study there appeared to be a fine balance between hyphal regrowth and sterilisation techniques, and the removal of contaminants while maintaining hyphal growth presented problems. In an attempt to remove bacterial contamination by *Bacillus* species observed using high power microscopy, tetracycline hydrochloride was also added to the medium. *Bacillus* species are amongst the most common of the soil bacteria (Vosátka, 1996), and this is likely to explain their predominance as bacterial contaminants of root pieces in this study. The inclusion of tetracycline as a sterilisation treatment did not prevent regrowth of AMF hyphae, but was similarly unable to completely prevent contamination unless streptomycin was included in the incubation medium. In all cases, sterilisation treatments appeared to retard hyphal growth, putting into question their applicability to studies of extra-radical hyphal growth. Previous authors have expended considerable research effort on the development of mycorrhizal root piece sterilisation techniques, and have still achieved only 20-40% success (Williams, 1990).

The specific aims of research work will dictate the requirement for the use of completely sterile culture techniques. Biochemical studies of aspects of the symbiosis are likely to be compromised by microbial contamination, because of the impossibilty of attributing observations specifically to the AMF, and therefore for critical studies aseptic growth of AMF is essential (Macdonald, 1981). However, relationships between AMF and other soil microorganisms are likely to be both important and complex, and unless the subject of research specifically requires their exclusion to enable specific aspects of the AMF themselves to be examined, their removal from the system will give an unrealistic account of the symbiosis. The existence of intimate bacterio-fungal relationships has recently been demonstrated. Both *Rhizobium* and *Pseudomonas* species have been shown to adhere to spores and hyphae of AMF germinated under sterile conditions. This interaction may be mediated through the production of cellulose-containing extracellular material of bacterial origin around the attached bacteria, suggesting the importance of either soluble factors or physical contact in interactions between AMF and rhizosphere bacteria. AMF are thus likely to be a vehicle for plant root colonisation by soil bacteria (Bianciotto *et al.*, 1996a). AM fungal cytoplasm has also been shown to contain bacterial endosymbionts. Bianciotto *et al.* (1996b) detected intracytoplasmic bacterium-like organisms (BLO's) in the spores and both germinating and symbiotic mycelia of *Gigaspora margarita*, suggesting an intimate relationship between AMF and soil microbial populations.

The host plant, AMF and microbial population probably make up a tripartite interaction in which each member affects the others in many and complex ways, both positive and negative (Vosátka, 1996). Stimulation of the external phase of AMF by

associated soil bacteria has been reported (Vosátka *et al.*, 1992), and bacterial influences have been found to be much more pronounced on the extra-radical mycelium than on the intra-radical hyphae (Gryndler & Vosátka, 1996). Both total and active length of the extra-radical mycelium were stimulated by bacterial treatments, while intra-radical infection was unchanged (Gryndler & Vosátka, 1996). These observations point to the questionability of using sterile systems in observations of extra-radical hyphal growth. In fact, it was the growth retarding effects of sterilisation techniques on hyphal regeneration from root pieces that resulted in a decision to use non-sterile inoculum in the present work. The growth of AMF in pure culture continues to be one of the most challenging goals of mycorrhizal research, and as such was unlikely to have been overcome as a subsidiary to the main objective of this research project.

Experimental Unit Design

Because of the difficulties associated with monoaxenic culture of AMF, the fungi are instead more often cultured in association with living plant roots. This introduces further contamination potential through the addition of plant-associated microorganisms, and has led to the development of experimental techniques using axenic root cultures as living substrates for the AMF (Wood & Cummings, 1992). Root organ and transformed root cultures have been used in attempts to identify *in situ* the compounds that are involved in the early stages of the symbiosis, or to assess the growth effects of various compounds on hyphal germination and development from spores (Bécard & Piché, 1989a & b; Chabot *et al.*, 1992). Such studies have generally concentrated on the establishment of the symbiosis. In the present study, microcosms were developed for observation of extra-radical hyphae in association with intact plants. Contamination problems, which impeded hyphal visualisation in the gel-based growth medium, were overcome when tetracycline was added to the sterilant. Initially, the aim was to study extra-radical growth from root piece inoculum separated from the plant root system by a membrane and a thin layer of gel-based medium as substrate. It was hoped that this would enable ease of hyphal visualisation through the removal of the membrane and hyphae from the microcosm for microscopic observation, while allowing effective plant-fungal communication. However, root piece sterilisation affected hyphal regrowth, as discussed above.

Following problems with hyphal growth from sterilised root piece inoculum, pre-inoculated sterile mycorrhizal plantlets were introduced into the microcosm system. Although the system was able to support the growth of both partners, the continuing development of the root system ultimately resulted in difficulties of hyphal

observation. This would have become particularly problematical in comparative assessments of imposed treatments, where detailed analysis of the hyphal growth form was required. In addition, assessment of growth effects occurring from application of specific test substances of plant origin would have been impossible to separate from other plant-related effects. For this reason, a technique for studying hyphal growth in the absence of a plant was developed. Similar plant-free systems have been employed by previous authors for examination of hyphal growth from spores (Siqueira *et al.*, 1982; Gianinazzi-Pearson *et al.*, 1989; Paula & Siqueira, 1990; Nair *et al.*, 1991). This work is described in detail in chapter 3.

This chapter aims to provide a background to the description and discussion of the work that follows, and to highlight some of the problems that are encountered in *in vitro* studies of AMF. In particular, the research carried out here faced specific problems associated with the study of the extra-radical phase of the symbiosis. The methods discussed here describe the evolution of the work as these problems were encountered and attempts were made to overcome them. This is picked up and carried through in chapter 3.

The author recognises that there are shortcomings associated with an experimental system for the study of an obligate symbiont which excludes the partner in the symbiosis. However, having chosen to study the plant-related factors which may affect the growth and development of that crucial component of the symbiosis, the extra-radical mycelium, it seemed necessary both to remove the influence of the whole plant to enable observation of the effects of specifically applied test substances, and to exclude soil related effects, which are described in more detail in section 3.2.3.

Chapter 3

3 Influences on Mycorrhizal Growth

3.1 Previous Related Work and Methodologies

Existing hyphal measurement techniques exhibit serious drawbacks, and thus quantification of length and activity of mycorrhizal hyphae has typically used methods which are both tedious and imprecise (Abbott *et al.*, 1992). Hyphal measurement is therefore an established problem area, and this is reflected in the number and variety of solutions that have been proposed over the years. These include the transformation of grid line intersections to hyphal lengths using stereological methods (Giovannetti & Mosse, 1980; Berg & Wessen, 1984); interactive measurement of an image by location of positions with a light pen and calculation of inter-point distances using computer software (Adams & Thomas, 1988; Packer *et al.* 1991); mycelial tracing using a pen and graphics tablet combined with measurement recording by computer software (Morgan *et al.*, 1991). Such methods as these can be inaccurate, over user-dependent and tedious, and these factors have combined to hold back research into hyphal growth. Estimates of the amount of AM fungal hyphae in soil vary greatly, both because of differences in experimental systems and because different components of the external phase have been measured (eg. hyphae attached to roots ± those extracted from soil; hyphal biomass; hyphal length per cm root). In addition there is generally no distinction made between active and dead hyphae (Sylvia, 1990).

3.1.1 Experimental Systems for Measurement of Hyphal Growth Parameters

There have been numerous studies of the hyphal growth form of both mycorrhizal and non-mycorrhizal fungi. Robinson (1973) reviewed a number of early methods; of these, the perforated plate technique was later used in modified form by Sherwood *et al.* (1992) in their studies of *Candida albicans*. This technique was used to study germ tube re-orientation on contact with perforations in sheets separating layers of spore suspension in nutrient agar from opposite layers containing specific chemical solutions. Sherwood *et al.* (1992) aimed specifically to study the growth orientation of *Candida albicans* in relation to topographical changes in the substratum. This type of contact sensing (thigmotropism) is an adaptation of organisms that live on or grow through solid surfaces. The germ tubes and hyphae of *Candica albicans* are apparently able to sense changes in topography and subsequently re-orientate their growth direction through 90^{o} in order to grow into the pores in the filter (Sherwood *et al.*, 1992). Gianinazzi-Pearson *et al.* (1989) similarly adopted a 2D system in their studies of the effects of host root exudates and plant flavonoids on mycorrhizal spore germination and early hyphal growth. In experiments

testing root exudate effects, surface sterilised seeds of both AMF host and non-host plants were germinated on agar in Petri dishes and grown for 7 days in the light. Seedlings were then removed from the dishes, and the agar inoculated with surface sterilised spores of *Gigaspora margarita*. The effect of flavonoids on the behaviour of *G. margarita in vitro* was also tested in Petri dishes by the addition of flavonoids to water agar at concentrations of 15nM to 15μM. Chemotaxic responses were examined by the introduction of the flavonoids into wells cut into agar medium 2mm from mycorrhizal spores. Both spore germination and hyphal growth of *G. margarita* responded positively to host root exudates, but neither were influenced by non-host root exudates. No changes in growth pattern or morphology of hyphae were induced, nor did these compounds act as chemotaxic attractants to *G. margarita* (Gianinazzi-Pearson *et al.*, 1989). Bécard & Piché (1989a) used dialysis membrane (molecular weight cutoff 12000-14000) to allow exudate diffusion from root cultures in a thin layer of overlying medium into the bulk of the agar medium below the membrane. Use of the membrane in this way facilitated removal of the roots to test exudate effects in the absence of the whole root. Similarly, Tatsumi *et al.* (1989) applied a two dimensional system to measurement of morphology in harvested root systems.

Recent work has been carried out with the non-mycorrhizal soil fungus *Trichoderma viride* Pers. on the application of image analysis (Section 3.1.2) in spatial characterisation of hyphal distribution within a mycelium. A non-uniform nutritional environment was created by placement of a $5mm^3$ agar block containing glucose, $NaNO_3$ and KH_2PO_4 as a point nutrient source. This block was placed in a Petri dish on a moist cellophane membrane inoculated with a 24 hour old germling colony of *T. viride*. Colony visualisation was achieved by direct projection onto lith film using a photographic enlarger (Hitchcock *et al.*, in press). In this work, the emphasis was placed on the techniques of image analysis necessary to assess mycelial growth. Membranes have also been employed to confine hyphae and prevent growth into semi-solid media. Allan (1983) used a cellophane membrane in this way in studies of the growth kinetics and branching characteristics of the filamentous bacteria *Streptomyces coelicolor* A3(2).

A second method which with various modifications has been widely employed in mycorrhizal research is the use of mesh in systems that contain roots while allowing hyphal penetration and outgrowth into a surrounding test medium. St. John and co-workers in particular have studied the extra-matrical hyphae of mycorrhizae to assess the distribution of total hyphal length and its relation to localised concentrations of organic matter. In these studies, chambers containing an inert medium (sandy nutrient-poor soil amended with 17μg g^{-1} phosphorus as hydroxyapatite) alone or

supplemented with organic matter were introduced into mycorrhizal pot cultures of *Fragaria* species grown from surface sterilised seed and inoculated with pre-germinated spores of *Glomus fasciculatum*. The inoculated host plants were placed in the experimental containers 92 days prior to the start of the experiment, and the response of the hyphae to placement of test organic materials (300 - 1000µm particles) determined by containing the particles within rigid containers separated from the soil by 45µm mesh screens. The experimental chambers themselves were constructed of polycarbonate, with one side consisting of 45µm stainless steel mesh through which the hyphae could enter the chamber. After incubation the length of absorptive hyphae in the chambers was determined by destructive harvest and measured using the line intercept method. Analysis of results showed a significantly greater absorbing length in the organic treatments than in the controls and indicated that the hyphae did associate preferentially with organic material (St. John *et al.*, 1983b). However, after further experimentation the hypothesis for a specific locational mechanism was rejected. A similar experiment using chambers of the same construction was carried out to assess both whether the organic matter particles themselves stimulated the increases in hyphal length, and whether encounters of hyphae with organic particles were more likely than with the control of sand only (St. John *et al.*, 1983a). The hyphae were found not to orientate specifically towards the organic matter particles and the greater hyphal length was accounted for by an increase in branching (St. John *et al.*, 1983a).

Hepper & Warner (1983), using sealed pouches of woven polypropylene fabrics penetrable only by hyphae, placed mixed inoculum from chopped infected *Zea mays* L. roots with sterile sand (sand only as control) in pots containing combinations of sterile sand and soil ± organic matter. The pouches were left *in situ* for 125 days, after which they were removed and the hole refilled with the appropriate substrate. White clover inoculated with *Rhizobium* was sown into the refill substrate 0, 10 or 50 days after removal of the pouches. After 98 days the clover roots were washed out of the soil, stained and assessed for infection to determine whether or not the initial inoculum had become established in the soil outside the pouch. A small proportion of soil in the medium was found to be necessary for the hyphae to grow out and establish a base. No infection was found in the indicator plants in pots where the outer medium was sterile sand, but amendment of the medium with organic matter, whether of soil origin or as peat, enabled hyphal colonisation and subsequent infectivity. The infective material appeared to be capable of independent growth, as infection of indicator plants occurred even when indicator seeds were not sown until 50 days after removal of the inoculum (Hepper & Warner, 1983).

Schuepp *et al.* (1987) also used the principle of differential mesh size to segregate roots and hyphae by constructing a segmented cuvette system in which the sections (a - c) were separated by "sandwiches" made up of single sheets of 80µm polyamide mesh between two sheets of 1mm mesh. The fine mesh physically restricted root growth between the cuvette sections while allowing the unrestricted passage of fungal hyphae. After three weeks, pre-inoculated clover plants were planted into each (a) cuvette to act as spreader plants, and clover seeds were sown into each (c) cuvette as receiver plants. A number of media were tested in the central (b) cuvette and were found to have a large effect on the spread of colonisation from donor to receiver plants. This was attributed to a number of factors including nutritive effects, pH, ease of penetration through the medium, textural differences, moisture content and possible indirect effects of the test substrate on production of hyphae by the donor plant (Schuepp *et al.*, 1987).

While these and other container systems which use mesh screens to separate hyphae from mycorrhizal root regions have been described, they allow mass flow of water and nutrient solutions as well as hyphal penetration. This means that a symbiosis-independent transfer of ions, particularly mobile ones, is likely to occur across the mesh barriers. In attempts to overcome this problem in studies of nutrient acquisition by mycorrhizal plants, Faber *et al.* (1991) used air gaps, paraffin wax or corn oil as barriers to uncontrolled nutrient transport, and Bethlenfalvay *et al.* (1991) used root free soil bridges between root and hyphal compartments. However, these methods were not ideal, allowing water loss to occur across the air gap, and mass flow across the root free soil bridges. In addition, the paraffin and corn oil barriers degraded causing oil seepage into the soil and leading to an alteration in soil moisture characteristics.

The use of hydrophobic microporous polytetrafluorethylene (PTFE) membrane (GORE-TEX®) with 5-15µm diameter pores has been recently suggested as an alternative for use in such experiments, as it can prevent uncontrolled nutrient transport by diffusion or mass flow between two soil compartments while still allowing penetration by mycorrhizal hyphae (Mäder *et al.*, 1993). Experiments using a saturated solution of KCl in the central compartment of a three part container, and distilled water in the lateral compartments, were run to test the permeability of the membrane to K^+ and Cl^- ions. No equalisation of conductivity in the lateral compartments occurred, indicating almost complete impermeability of the membranes to these ions. GORE-TEX® PTFE membranes are laminated onto a polyester support (product information Gore & Associates GMBH, Germany) which Mäder *et al.* (1993) faced towards "donor" plants inoculated with *Glomus mosseae* in a further

experiment to test penetration of membranes by mycorrhizal hyphae. "Receiver" plants (non-mycorrhizal) were grown in sterile soil at the PTFE side of the membrane. Stained root samples of acceptor plants assessed for mycorrhizal infection using the grid line intersect method showed rapid colonisation by AMF, with a continuous increase to 60% after 13 days. This colonisation level was similar to that observed across 60μm nylon mesh (Mäder *et al.*, 1993).

While these methods provide a satisfactory way of separating root and hyphal growth, harvest is destructive and thus the systems cannot be used in specific determination of hyphal distribution. The delicate nature and inaccessibility of the mycelial network make non-destructive investigation difficult and root observation chambers have therefore been used to permit *in situ* examination of mycelial development and function in near natural conditions. Finlay & Read (1986) used this type of system, which allows the spread of mycorrhizae between inoculated donor plants and non-inoculated receiver plants to form a network of interconnecting hyphae, to study the structure and function of the vegetative mycelium of ectomycorrhizal plants.

Similar root observation chambers were adopted by Friese & Allen (1991) to study the growth dynamics of AMF hyphae from inoculum source to roots, and from the colonised root out into the soil matrix. The chambers were made up of two 12 x 30 cm glass plates separated using a 5mm layer of silicon adhesive. A 2cm band of inoculum was placed 10-20cm below the surface of steam sterilised soil in each chamber. Pre-germinated seeds were sown into the chambers seven days after inoculation. The chambers were tilted at a slight angle to allow some of the roots to grow against the lower glass plate thus increasing ease of observation, and were examined at 5 day intervals under a dissecting microscope. Growth of hyphal networks against the glass enabled direct observation of development. The external hyphae were observed to extend up to 10cm beyond the inoculum layer and consisted of both runner hyphae, apparently responsible for secondary infection, and an absorptive hyphal network which developed into a fan of dichotomous branches. These fan shaped networks projected on average 5cm from the root surface, and died back after approximately 5-7 days (Friese & Allen, 1991). The study concentrated largely on the external hyphal architecture, but although they commented on the efficiency of the absorptive hyphal networks at exploiting the soil matrix for nutrient and water acquisition, they did not attempt to define the specific distribution of these networks in space.

3.1.2 Interpretation and Quantification of Results: Image Analysis and Fractal Dimension

3.1.2.1 Image Analysis

Image analysis has many and varied applications. Its early usage was primarily in metallurgical laboratories for quality control and other microstructural measurements, but its applicability to other fields soon became apparent. Its applications have included volume analysis of fractions of alloys and geological samples, number and size analysis of particulate contaminants filtered from air or fluids, and distribution of optical densities in nuclei. It also has a wider role in, for example, interpretation of satellite images.

The morphological characterisation of commercially important filamentous fungi and *Streptomycetes* is useful in fermentation studies and is necessary for the design and operation of fermenters. Because of this, image analysis systems capable of undertaking such morphological measurements have been developed. Image analysis is the science of making geometric and densitometric measurements on images from any source. Its main application is in quantitative microscopy to give rapid and accurate data generation in place of more traditional subjective methods. Image analysis systems use a camera to transfer optical images from a microscope or macro viewer for subsequent conversion into an electrical signal for processing. The video signal of the field of view is sent to a computer capable of image processing and analysis, and the image is digitized to produce an array of picture elements (pixels). Once an image is captured in digital form it can be processed to improve quality or to select specific features, and then analysed to obtain the required measurements (Packer & Thomas, 1990). The first image to be analysed is stored in digital form and is then "segmented" to define the grey levels which are of interest. Image processing is performed on the grey image to enhance it by picking out detail or removing unwanted artefacts. A binary image which shows the selected objects is then defined by thresholding, and subsequent processing is carried out on this image. Objects other than those to be analysed, which are likely to be present as contaminants within the field of view, can thus be eliminated. Where the object of interest is filamentous, such unwanted contaminants can, for example, be removed using circularity parameters.

Hyphae often appear thin and blurred, and images are therefore composed of variable intensities. A threshold is chosen to separate the mycelium from the background, and any pixel with a value greater than that of the threshold is assumed to lie on the mycelium. If the threshold is set too high, very thin hyphae may be missed; if too low, there is a risk that hyphae in close proximity to each other will

become joined in the binary image. The easiest way to extract the mycelial structure is to thin the binary image by the process of skeletonisation, which selects pixels medial between the edges of selected objects. This results in a skeleton of the original image which retains its topology but is reduced to a single pixel width. Basic structure is extracted on the principle that any pixel with only one neighbour in the skeleton is the endpoint of a hypha; any with three or more neighbours forms part of a junction. It is not possible to tell from the skeleton whether hyphae meeting at a junction are joined, or whether they are crossing over. Pixels with two neighbours form chains which connect junctions and endpoints. These "rules" allow the interpretation of any image which is basically a network of lines (Hitchcock *et al.*, in press).

Tucker *et al.* (1992) presented mycelia on slides under coverslips and examined them microscopically using a Quantimet 570 (Leica, UK) linked via a colour video camera. Image analysis routines were similarly selected and combined to develop software suited to the morphological characteristics of mycelium. Image analysis of hyphae mounted on slides also requires that they are harvested destructively. Recent work by Hitchcock *et al.* (in press) approaches the use of image analysis in quantifying fungal response to a heterogeneous nutrient environment. Colonies of the soil-inhabiting fungus *Trichoderma viride* were visualised by direct projection onto lith film using a photographic enlarger. An enlargement of this high contrast image was then digitised for subsequent analysis.

Image analysis techniques may vary from fully manual to fully automatic. Silage & Gill (1984) discussed an almost fully manual application developed for morphometric point counting tasks, which allowed manual tracing of boundaries using an interactive device. Hand drawings of this type do have an intrinsic problem of user "jitter", producing jagged tracings which necessitate the subsequent application of smoothing techniques to remove "noise" from the image (eg. Deoras *et al.*, 1990). So-called "control points" (chosen points in the image, such as the starting points) are commonly used to shape the edge of an object interactively. These work by either locating a strict path through which the traced edge must pass, or by controlling the shape of a curve in a predictable way. According to Freeman (1978), critical points in classical geometry are maximum and minimum points and points of inflexion. This has generally been expanded to include discontinuities in curvature, endpoints, intersections (junctions) and points of tangency. Metz *et al.* (1981) introduced a semi-automatic method for the quantitative representation of mold morphology using control points for recording five independent features of topological interest. These were the length of the main hypha, hyphal diameter, length of branches, length of segments (tip to first branch point) and number of branches. Measurement of these

features used an algorithm for which a number of control points were located using a light pen. All measurements were taken as straight line distances between these points, and proved reproducible for continuous but not batch cultures.

Control points can be used to mark a starting point (eg. Capowski, 1989). A small arc centred on the starting point can be scanned and a grey level for each point on the arc recorded, which forms a waveform of intensity across the hyphae. The segment is located by choosing the point which indicates a dip in the waveform representative of a change in grey level intensity. This point is chosen as the centre of the dip location, and the algorithm is therefore able to follow the hyphae recording its position. When the algorithm reaches a branch point, a double dip will appear in the waveform. The algorithm will record the branch point and then arbitrarily direct tracing down one branch pathway before returning to that point and recording the other. Inevitably, even with a good set of images, the algorithm may become confused, finding it impossible to determine parts of the structure. This is more particularly the case with complicated images such as the AMF mycelium under consideration in this study, which have little distance between branch points, and which in their original form also have considerable background noise problems. For more complicated images, further user intervention such as manual editing by deletion or additional tracing may therefore be needed (Iain Inglis, pers. comm.). Supposedly fully-automatic algorithms do exist, but in reality those for all but the simplest raw images require user intervention to achieve any level of success. User modification can ensure that the correct pathway is chosen or correct any mistakes, and since all results can be performed and viewed on-screen, this can be feasible in real time.

3.1.2.2 Fractal Dimension

Euclidean geometry is motivated by the desire for simplicity and order in nature, which inevitably results in approximations and caricatures. Whether nature is essentially either complex (irregular and random), or simple (ordered/Euclidean) is an artificial dichotomy, and poses the question as to whether the appropriate geometry can combine complex patterns and simple descriptions. The concept of fractals may bridge this gap. Natural patterns can be very complex, but they also appear to be scale invariant ie statistically unchanged under magnification or contraction over a wide range of scales. The essential feature of fractals is the way that the material composing them is distributed in space - clustered heterogeneously but not randomly such that the structure looks statistically identical independent of the scale at which it is viewed. In practice, there may generally be several scaling regions separated by breakpoints, with scale invariance holding for each region but failing when the

breakpoint is crossed. Scale invariant objects are termed fractals. The key concept underlying fractal geometry is self-similarity, which is the ability to decompose into smaller copies of itself ie. the property by which the structure of the whole is composed of its parts, making it possible to rebuild the whole out of highly magnified portions of itself. Fractals may be *regular* if they are self similar ie. may be written as a union of rescaled copies of themselves, and rescaling is uniform in all directions, or *random* if they are self affine ie. rescaling is direction-dependent. Almost all natural fractals are random.

Irregular objects cannot be reduced to the usual building blocks of line segments, squares and cubes. Magnification reveals the same irregularity on small scales as on large. Complex objects, fractals, cannot therefore be rigorously measured by the basic building blocks of Euclidean geometry. The basic building block of a self-similar object (irregular form), is an infinitesmally small copy of itself. Measures of complex objects can thus be carried out by enumeration of these irregular building blocks. In Euclidean geometry, the basic building block is simple, but its use for the description of complex forms is complicated by attempting to force the simple block into the measurement of the complex form. The length of a line, for example, consists of the length of the unit used to measure it, multiplied by the number of times the unit can be placed along the length. In Euclidean geometry, if the unit size is halved, twice as many units are required to span the length. In general, the number of units $n\ (l)$ is related to the size of the unit l by the relation:

$$n\ (l) \approx 1/(l/l_{max})D$$

D = Euclidean dimension (in this case 1)

l_{max} = largest l spanning line

This relation extends to 2 and 3 dimensions when D is equal to 2 and 3, and where l is the length of the side of tiles which tessalate a plane, or the edge of boxes which fill a volume. When concerned with shapes that fill space with smoothness intermediate to Euclidean forms, the above equation can be generalised to hold for fractional values of D. Such shapes are known as fractals, and D becomes the fractal dimension (Crawford *et al.*, 1993). In fractal geometry, the complexity comes from the building blocks, while the process which generates the larger pattern is relatively simple (recursion). The repetition of a simple rule gives us self-similar geometry.

Natural patterns, particularly in ecosystems, frequently appear irregular, complex and hard to measure even at the small scale. Self similarity forces the

complexity of the object into the building blocks and describes the inherent irregularities through power laws (Mandelbrot, 1977 & 1982) or scaling rules. These are characterised by scaling exponents which are constant within each scaling region but jump at the breakpoints between regions. Many regular geometric figures are self similar, and self similarity has its origins in formulae for the area of a square and volume of a cube.

Area of a square: $A=S^2$

Volume of a cube: $V= S^3$

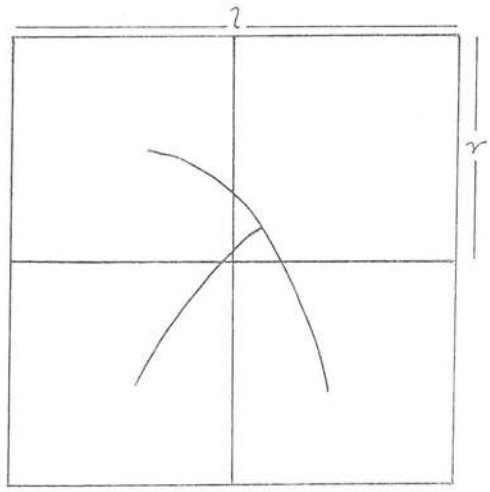
The power laws are simple where the measured parameter is an integer as they follow from decomposing a square of side S into S^2 similar smaller unit squares, and a cube into S^3 smaller unit cubes. Area and volume formulae relate the size of simple objects to their linear scales, with the *dimension* serving as the key parameter. The formulae follow from decomposing the objects into unions of similar smaller objects. Cubes, squares and line segments are therefore self similar, and the dimension is called the scaling or similarity dimension. The scaling dimension measures the "mass" of a self similar set x in terms of small scale copies of x. A point is trivially self similar with a scaling dimension of 0. The power of the concepts of self similarity and scaling dimension become apparent when they are used to characterise complex irregular objects. Assuming that x can be decomposed into n rescaled copies of itself, each contracted by a linear factor of k, then

$$D=\log n/\log k$$

A regular polygon (or a straight line) has a dimension (D) of 1, while a filled in planar figure (or a plane filling curve) has a dimension of 2. The power laws provide the fundamental algebraic property that links dimension and self similarity.

The heterogeneity of natural systems means that it is not possible to express meaningful averages, and fractal geometry has been seen as a basis for studying dynamics in heterogeneous media (Crawford *et al.*, 1993). Tatsumi *et al.* (1989) used the concept of fractal geometry in measurement of plant root system morphology. Plant roots form a complex system of branches within the soil matrix which is important in terms of root function (eg nutrient and water uptake). Being able to quantify root morphology, particularly with respect to branching, is important in analysis of architecture and in understanding the contribution of the root system to plant growth. Like other branched systems, roots should be amenable to fractal modelling (Tatsumi *et al.*, 1989). Root systems were photographed and analysed in

the 2D form. The photographs were converted to video signals by a TV camera connected to an image processor unit, and a grey image (based on 256 levels) generated in the memory frame (512 x 512 pixels). After filtering and enhancing the edges, the image of the root was discriminated from the background as a set of pixels of full grey level. A large square frame was placed over the image, and divided into $(l/r)^2$ smaller squares, each of side r. Thus, the image was digitised by pixels of given scaling factor r, indicating that the generated image was divided by the pixels, each of which corresponded to a small square of side length r (Figure 3.1).



Number of small squares = $(l/r)^2$

Number of squares intersecting object = N (r)

Figure 3.1: Description of radii for fractal analysis of plant root systems

The number N (r) of pixels making up the root image was counted, and the scaling factor r of the pixels recorded. Measurement of N (r) at larger scaling factors (lower resolutions) was done by zooming down the image with four adjacent pixels making up one pixel. Measurement at higher resolutions was done by a close-up with several images necessary to cover the whole root system. This technique was automatic.

If log N (r) at small values of r plotted against log r gives a straight line with a negative slope (-D), the object is fractal, and D is the fractal dimension. Root systems were found to be self similar within the scale range approx. 0.3-20 mm. Root system morphology could be characterised by the slope of each line as an estimate of the fractal dimension D. Higher values of D represent greater intricacy (ie.a greater degree of branching). From their work Tatsumi *et al.* (1989) concluded that for the species tested, plant root systems have a fractal structure and that data required for the analysis could be obtained by image processing.

Similarities in growth form make it possible to analyse fungal hyphae in a similar way, and there have also been a number of studies of fungal hyphae based round the concept of fractal analysis. Quantitative fractal geometry can yield insights into the mechanisms whereby spatial organisation influences the interaction between structure and biotic processes in the soil. Ritz & Crawford (1990) examined the growth of *Trichoderma viride* on cellophane over a homogeneous substratum, and hypothesised that the fractal dimension reflected the compromise between exploitative and explorative growth forms. When the nutrient status of the substrate was poor, the colony formed a low dimensional morphology ie. minimum hyphal mass distributed across maximum area. On encountering a nutrient concentration, the dimension increased towards 2, and filled space as effectively as possible to exploit the new substrate source.

True complexity is information intensive, but increases the possibility for adaptation to a dynamic environment. Self similarity may be the key to generating complex structures from minimum coding (Ritz & Crawford, 1990). The fractal concept thus provides a powerful insight into the quantitative aspects of the space filling properties of fungal hyphae. Ritz & Crawford (1990) used this concept to examine the growth of *Trichoderma viride*, basing the work round a number of equations:

(1) $M(r) = kr^D$

M (r) = mass contained within radius r

k = constant

D = constant

Equation $1/\pi r^2 \Rightarrow$ surface density (ρ) of structure

If D = 2, ρ is constant and implies that the material has an homogeneous planar distribution.

If D < 2, ρ decreases as r increases, describing a more open, clustered distribution. The distribution is fractal, and D is the fractal dimension.

Equation (1): the total hyphal mass within a region is controlled by k and D, which together specify how the material is organised within r.

Exponent D describes how the mass is distributed across small and large scale structure ie. how branched the colony is.

k fixes how much mass is thus distributed.

The total contained mass increases more slowly with the radius r for a small value of D than for a large value. A given amount of mass can therefore be distributed across a

larger area for a small D than for D≈2. D indicates the distribution of mass (as branching). If D is small, ie. relatively unbranched, the mass increases slowly with radius, therefore a given amount of mass is distributed across a larger area. An unbranched colony is inefficient at gathering nutrients because it occupies only a fraction (δ) of the available space.

$$(2) \qquad \delta = k/\pi\rho m \; R^{D-2}$$

Colony size R on 2D surface with mycelial surface density written as ρm

The space filling efficiency falls for D progressively <2, and reflects the compromise between exploration and exploitation strategies.

In their examination of substrate exploration by *Trichoderma viride*, Ritz & Crawford (1990) analysed photographic enlargements of colonies. For fractal analysis, the total hyphal mass (assuming a constant mass:length ratio) was determined as a function of the distance from a point in the middle of the structure.

Value D was obtained from linear regression analysis of the plot of ln M (r) against ln r. The slope of this line corresponded to D, and the intercept to ln k. For larger values of r, data would fall below the extrapolated linear relation if the structure was fractal. Ritz & Crawford (1990) obtained data consistent with the power law relationship between the mass and the radius (Equation (1)). D was always found to be less than 2 (the mass increased more slowly with the radius, indicating a lower space filling efficiency) but the data supported the hypothesis that growth was fractal. Colonies apparently evolved from being fractal, to being non-fractal with homogeneous distribution of mass over time. Ritz & Crawford (1990) suggested that optimisation of nutrient capture by colonies was achieved by optimisation of the balance between the exploratory and exploitative modes of growth. Thus, following germination growth will be largely exploratory (low D value), with a given mass distributed over a larger area. More efficient exploitation of space is necessary when nutrients are present (this requires an increased value of D). This is achieved through branching, and growth becomes exploitative.

There is no requirement for exploratory growth where the nutrient supply is unlimited. The colony can thus continue to branch until D=2 (ie homogeneous distribution of mass). The apparently coherent growth of *Trichoderma* is unlikely to be an illusion created by the spatial homogeneity of the nutrient, because of the heterogeneity observed in the fractal structure (Ritz & Crawford, 1990). The fractal structure implies no preferred centre to the colony - all points within the structure are

at the centre of the same hyphal mass distribution. For a colony growing in heterogeneous conditions, any part of the colony encountering a concentration of nutrients will absorb these, establishing a nutrient gradient within itself with the maximum at the site of concentration and increasing the likelihood that branches will be initiated in the part of the colony located at the resource itself. Fungal behaviour under heterogeneous conditions will thus be coherent upto the point at which significant fluctuations occur in the substrate. The demonstration of fractal growth patterns is important. Quantifying the fractal dimension of mycelia provides a powerful tool in the quantification of space filling characteristics during the developmental stages of the mycelium (Ritz & Crawford, 1990).

To date, no similar work has been carried out on hyphal distribution of AMF using image analysis techniques and computation of fractal dimension (FD). Adoption of methods previously designed for other species of fungi has the potential to provide important information concerning the growth characteristics of AMF, and how these may change in respose to environmental stimuli.

3.2 Responses to the Environment

3.2.1 Plant Signals

Plant root systems play two main contributory roles in the growth of AMF, acting firstly as an immediate trigger providing important stimulation for hyphal growth from the spore, and maintaining growth once initial and crucial spore reserves are depleted. A second phase of fungal growth is then initiated which is independent of the spore and which becomes established as the first arbuscular component of the colonisation develops.

These two mechanisms are nutritionally differentiated, the first fuelled by reserves from the spore, and the second supported by the host plant. Activation of the first occurs prior to fungal contact with the root, but also ceases in the absence of the root. In general, plant root exudates do not improve spore germination, but can stimulate hyphal growth from spores (Bécard & Piché, 1989a). Thus, early interactions between AMF and host involving root exudates are exhibited after spore germination (Azcon & Ocampo, 1984; Bécard & Piché, 1989a). The suggestion has been made by several authors that specific compounds contained in root exudates are capable of stimulating hyphal growth of AMF (eg. Elias & Safir, 1987; Mosse, 1988). Root exudation must provide an important influence on mycorrhizal development, particularly during the first stages of fungal growth, as root metabolites may be implicated in sustaining fungal growth during pre-infection stages. There has been

considerable interest in evaluating these and other relationships between plant roots and rhizosphere organisms.

Work on zoosporic fungi using culture-grown roots has suggested that taxis to roots is generally not host specific ie. fungi react to common components of diffusates (Deacon & Donaldson, 1993). Taxis *in vivo* may therefore involve combinations of attractants, allowing assured host contact even if receptors for individual compounds become saturated (Carlile, 1983). Some *Phytophthora* species have recently been shown to respond to specific compounds at low concentrations *in vitro* (Morris & Ward, 1992), but the significance of such host specific compounds has not yet been determined. Studies with bacteria do however suggest that a high-affinity chemotactic system for specific compounds may be superimposed over a more general response to root exudates (eg. Caetano-Anolles *et al.*, 1988).

For AMF it has been suggested that barriers to colonisation in non-host plants are probably physical, and not related to exuded compounds. The formation of entry points does not signify that a successful association will be formed, and Ocampo *et al.* (1980) observed that in *Brassica* - *Glomus* interactions the majority of appressoria failed to penetrate the roots successfully. This may implicate characteristics of the root epidermis or cortex in preventing the development of infection, rather than the action of compounds produced by the roots (Ocampo *et al.*, 1980). The considerable development of AMF hyphae observed around the roots of non-host plants, together with the presence of aborted entry points, also implicates a physical rather than a chemical barrier to non-host colonisation (Ocampo *et al.*, 1980). A lack of evidence for the production of inhibitory substances by non-host plants (Ocampo *et al.*, 1980; Glenn *et al.*, 1985) similarly indicates a physical rather than a chemical barrier to infection.

In some instances, non-host *Brassica* plants have been observed to show some degree of mycorrhizal infection when grown in the presence of a host plant, failing to reveal any production of mycorrhizal inhibitors and raising the possibility that AMF may derive some benefit from the presence of non-host plant roots (Ocampo *et al.*, 1980). However, infection was generally <5% of root length, and due to lack of arbuscle development appeared atypical. Ocampo *et al.* (1980) postulated that the inability to infect observed with *Gigaspora margarita* compared with *Glomus fasciculatum* "E3" may be accounted for by its primarily arbuscular state and tendency not to form vesicles in typical host plants. Glenn *et al.* (1985) similarly found with *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe that no penetration of non-host roots occurred. In comparison, *Glomus mosseae* initially appeared able to penetrate roots of all tested *Brassica* cultivars. However,

ultrastructural studies did not support this observation in roots with intact epidermal tissue, showing that no intracellular growth was observed in *Brassica* and that penetration appeared to be successful only in dead root cortical cells (Glenn *et al.*, 1985). Thus, while vigorous hyphal growth occurred in *Brassica* roots with dead epidermal and cortical cells, hyphae in the vicinity of healthy *Brassica* roots appeared senescent, indicating the failure of these non-host plants to support functional AMF (Glenn *et al.*, 1985). This work suggests that non-colonisation of *Brassicas* by AMF is due not to the presence of inhibitory factors produced by the plant, but to the lack of stimuli necessary for penetration and arbuscular development (Glenn *et al.*, 1985).

Although non-host plants have an obvious impact on mycorrhizal associations, it appears that non-host crops do not suppress colonisation by AMF in following host plant crops (Ocampo & Hayman, 1981). Observations have shown that AMF hyphae can grow in the vicinity of non-host plants (Ocampo *et al.*, 1980) and it has been suggested that these hyphae are more active in infecting roots than fresh inoculum (Ocampo & Hayman, 1981). The build-up of AMF colonisation in barley plants occurred much more rapidly when roots from a previously harvested oilseed rape plant were left in the soil than after fallow or the addition of fresh inoculum, suggesting an increase in inoculum potential of AMF despite close contact with the roots of non-host plants (Ocampo & Hayman, 1981). This is possibly because non-host root exudates do not affect spore germination (Ocampo & Azcon, 1980) and suggests either that inhibition by non-host root exudates is not occurring, or that host factors are able to overcome non-host suppressants. Plant interactions are likely to have an important role within a community, as single species populations are rare at least in natural or semi-natural environments.

However, contradictory evidence has been obtained, and non-host inhibition of colonisation by AMF has also been observed, implicating non-host exudates in non-colonisation and in depression of host plant colonisation in mixed populations (Hayman *et al.*, 1975; Iqbal & Qureshi, 1976). Non-host plants may have intrinsic factors implicated in the absence of the development of AMF infection in their root systems (Ocampo *et al.*, 1986). The production of toxic exudates by non-host roots cannot be discarded. Alternatively, it may simply be that non-host plants do not exude stimulatory compounds (Gianinazzi-Pearson *et al.*, 1989).

Plants are able to synthesise secondary compounds which can profoundly influence other organisms in the environment. Phenylpropanoid-derived metabolites are widely distributed in the plant kingdom, and have many functions. They are implicated in chemotaxis, *nod* gene activity and plant protection against pests, diseases and damage by UV light, and also have a determinant role in flower colour

and attraction of insects for pollination (Graham, 1991; Kape *et al.*, 1992). Because of the vast structural diversity and complexity of distribution of metabolites and their derivatives, it also seems likely that they may have additional roles and bioactivities. Phenolics are amongst the most widespread of the secondary metabolites, and do appear also to play important roles in plant-microbial interactions. There is increasing evidence that flavonoids may be implicated as signal molecules in plant-fungal relationships.

Although the understanding of the molecular mechanisms of communication between AMF and host plants is not as advanced as that between *Rhizobium* and legume hosts, the assumption is that similar mechanisms are involved (Kape *et al.*, 1992). Root infection by *Rhizobium* species proceeds in distinct phases, each influenced by complex spatial and temporal interactions with specific host tissues at defined developmental stages, each of which may involve exchange of specific signal molecules between the host and symbiont. Plant flavonoids are an important class of signal molecules in legume nodulation; host-adapted *Rhizobium* species show both taxis and activation of nodulation genes in response to host flavonoids (Djordjevic & Weinman, 1991), and the effects are specifically blocked by flavonoids of non-host plants (Caetano-Anolles *et al.*, 1988). Structural *nod* genes are induced by different sets of flavonoids, as different *nod*D proteins have different inducer specificities; a protein from a given *Rhizobium* species is generally most active in the presence of exudates from plants nodulated by that particular species (Spaink *et al.*, 1989). In general, these proteins can be activated by the presence of a family of related flavonoids. Those from narrow-host-range rhizobial types, such as *R. meliloti*, *R. leguminosarum* and *R. trifolii* will respond to few flavonoids, and vice versa. The activity of a given flavonoid will similarly vary with the species of *Rhizobium*. Different flavonoids in particular exudates can interfere with the ability of others to induce *nod* gene expression. The spatio-temporal distribution of flavonoids both in the rhizosphere and at the root surface is likely to determine the level of induction of the associated rhizobial *nod* genes (Dénarié *et al.*, 1992).

Flavonoids are also thought to be closely implicated in the mycorrhizal symbiosis. Common flavonoids have been shown to increase the rate of spore germination and stimulate hyphal growth of AMF from spores of *Gigaspora margarita* (Gianinazzi-Pearson *et al.*, 1989). Hyphal growth from *Gigaspora margarita* spores has also been shown to be considerably stimulated by flavonol aglycones (Bécard *et al.*, 1992; Chabot *et al.*, 1992) and root exudates (Bécard & Piché, 1989a). The physiological basis of these growth promoting effects has not been elucidated, although it is possible that general activation of the phenylpropanoid

pathway, which is also responsible for the production of other promoter compounds, may favour AMF-plant interactions (Gianinazzi-Pearson *et al.*, 1989).

Examination of the occurrence of flavonoids and isoflavonoids in soyabeans was prompted by their role in chemoattraction and nodulation in rhizobial associations. Rapid secretion of conjugates of the isoflavones daidzein and genistein into seed and root exudates of soyabeans at levels consistent with chemotaxis or induction of nodulation genes, indicated that these compounds may play a constitutive role in resistance of particular seedling tissues to disease (Graham, 1991). The distribution of phenyl-propanoid metabolites in soyabean tissues has been linked to molecular interactions with both potential pathogens and symbionts, and is likely to be important in determination of molecular mechanisms for the interaction of the plant with microbial associates (Graham, 1991).

Transformed roots have very low nutrient requirements, and this permits their culture in media also appropriate for fungal growth, thus allowing more detailed studies to be carried out. Both organic extracts and exudates of tranformed roots have shown similar flavonoid patterns. Bel-Rhlid *et al.* (1993) found that of compounds detected in extracts and exudates of Ri T-DNA transformed roots of carrot, quercetin and kaempferol were stimulatory to growth of *Gigaspora margarita*, chrysin and apigenin inhibitory, and rutin and luteolin ineffective. Quercetin, the most widely distributed flavonoid in mycotrophic plants, was found to be the most abundant. The presence of flavonoids in transformed roots and exudates is significant in their role as molecular signals during early establishment of the mycorrhizal symbiosis (Bel-Rhlid *et al.*, 1993). Hyphal growth from spores of *G.margarita* is affected by both stimulatory and inhibitory flavonoids commonly found in plants, and quercetin is the most potent for stimulation of its growth (Chabot *et al.*, 1992). Siqueira *et al.* (1991) tested the effects of flavonoid compounds on growth of *Trifolium repens* inoculated with *Glomus* species, and found evidence for concentration dependent increases in root colonisation and growth of the clover seedlings as a result of applications of the isoflavonoids formononetin and biochanin A and the flavone chrysin. Similarly, Kape *et al.* (1992) found that concentration of flavonoids was important in biological response. The mechanisms of this response have not been determined, but may be mediated by changes in membrane permeability, enzyme activity or DNA transcription.

The release of isoflavones has been posed as a specific and regulated phenomenon, although the precise role of these metabolites as possible signal molecules has yet to be established (Graham, 1991). It seems certain that flavonoids are fundamental regulatory factors in the early events which lead to the establishment

of the symbiosis, and recent reports suggest that flavonoids alone amongst constituents of root exudates modify the rates of spore germination and hyphal growth (eg. Gianinazzi-Pearson *et al.*, 1989; Nair *et al.*, 1991; Chabot *et al.*, 1992).

However, while flavonoid analysis may have increased the understanding of interactions between plants and both pathogens and symbionts, the question remains as to their precise role and occurrence as regulatory plant metabolites in AM associations in nature. Although there is well documented evidence for their close involvement in the mycorrhizal symbiosis, other authors provide contradictory evidence, and suggest the importance of both whole root exudates and alternative components as regulators of the plant-AMF interaction. In addition, other non-chemical factors which affect the rate of root extension, such as light, soil temperature or plant development stage have important roles in mycorrhizal development (Azcon & Ocampo, 1984). The use of controlled conditions may mask real life effects. Under natural conditions other environmental factors such as soil type and the composition of the rhizosphere microbial population may influence the relationships between the partners in the mycorrhizal symbiosis and other soil organisms (Ocampo *et al.*, 1986).

Chemical analysis of exudates of Ri T-DNA transformed carrot roots found to be stimulatory to the growth of AMF did not show the presence of flavonoid components. Results of this work indicated that other root metabolites, such as derivatives of caffeic acid, may be stimulatory to growth of AMF, and that flavonoids are not necessary for the establishment of the mycorrhizal symbiosis (Bécard *et al.*, 1995). Not only may alternative rhizosphere components play an important role in the mycorrhizal symbiosis, but synergistic effects between different groups of compounds may also be critical to stimulation of fungal growth. It has been suggested that the simultaneous presence of root volatiles and exudates may be responsible for fungal growth stimulation, and that in the absence of root volatiles fungal growth with or without exudates may not differ significantly. Volatiles may thus promote hyphal elongation, acting in synergy with exudates to increase the initial rate of fungal growth. Volatile compounds are likely to play a significant role *in vivo* because of their capacity to diffuse rapidly over long distances. It is likely that CO_2 is the most important constituent of the volatile fraction, as CO_2 trapping results in cessation of hyphal growth (Bécard & Piché, 1989a).

Sugar content is also likely to be an important factor in assessing the contribution of root exudates to the success of the mycorrhizal symbiosis. Azcon & Ocampo (1984) found that in the early stages of plant growth there was no apparent relationship between the degree of colonisation by AMF and the total sugar content in the root exudates of several plant species of different mycorrhizal susceptibilities.

Root exudates from host and non-host plants influenced spore germination under controlled conditions to similar extents; non-host plants showed a higher exudation ability during early growth than did host plants. This raises the question as to whether sugar leakage from roots is related to the extent of mycorrhizal infection, and to the mycotrophic status of a given plant species.

In this study, Azcon & Ocampo (1984) found no relationship between the infected root length and the total sugar content of the exudates. Non-mycorrhizal plants (both non-host and non-colonised host) exuded more sugars. The sugar content of root exudates has an important influence on mycorrhizal colonisation as there appears to be a direct correlation between the level of colonisation and the quantity of sugars exuded. Lack of mycorrhizal colonisation in non-host species has previously been attributed to low sugar levels in their root exudates (Schwab *et al.*, 1982) rather than because of any detrimental effect on the fungus (Ocampo *et al.*, 1980), suggesting that root exudation is a critical factor in controlling AMF formation. However, it appears that the amount of sugar exuded may not be a decisive factor for the initiation of infection, and that non-infection in non-host plants is not because of sugar exudation insufficient to sustain initial fungal development (Azcon & Ocampo, 1984). On a weight for weight basis, exudation in susceptible plants is less than in non-susceptible, which indicates that differences in root permeability may exist in early phases of growth. Exudation rates appear to decrease with time in non-host plants and increase in host plants, emphasising the importance of temporal as well as chemical differences in factors which may affect the development of the symbiosis. If plant susceptibility to AMF is not dependent on the amount of sugar exudation, alternative factors such as root membrane constitution and/or the presence or absence of root metabolites are likely to be involved.

3.2.2 Chemotactic, Chemotropic and Foraging Responses

Chemotaxis in natural systems is a widely occurring phenomenon. It is thought that rhizobia are able to respond by chemotaxis to plant root exudates and move towards localised sites on legume roots (Gaworzewska & Carlile, 1982; Gulash *et al.*, 1984; Dowling & Broughton, 1986). *Rhizobium* species are attracted by potential nutrients, but also by compounds not necessarily of nutritional value, such as flavonoids or their precursor cinnamic acid (Aguilar *et al.*, 1988; Kape *et al.*, 1991; Kape *et al.*, 1992). Collection of root exudates from soya bean roots has suggested that daidzein and genistein conjugates are rapidly released from roots at levels consistent with a potential role in rhizobial chemotaxis (Graham, 1991). Such chemotaxis towards flavonoids may facilitate contact between the bacteria and the

root surface of a specific host, and host-adapted *Rhizobium* species are known to show taxis and activation of nodulation genes in response to host flavonoids (Djordjevic & Weinman, 1991). The effects are specifically blocked by flavonoids of non-host plants (Caetano-Anolles *et al.*, 1988).

Chemotaxis also occurs widely in pathogenic fungi. The main infective agents in plant diseases caused by the fungal pathogens *Phytophthora* and *Pythium* are zoospores. These are motile propagules which are attracted chemotactically to plant surfaces, often very strain-specifically. The isoflavones daidzein and genistein are highly chemoattractive to zoospores of *P. sojae* Kauf. & Gerd., which is an economically important pathogen of soyabean (Morris & Ward, 1992). These simple isoflavones are also inducers of nodulation genes in the nitrogen-fixing bacterial symbiont of soyabeans, *Bradyrhizobium japonicum* (Section 3.2.1). Thus, both the pathogen and the symbiont identify their host by recognition of the same chemical signal (Morris & Ward, 1992).

Zoosporic fungi are taxonomically diverse, extending to a wide range of habitats and with diverse activities ranging from saprophytism through commensalism to aggressive pathogenicity. Justification for this broad fungal grouping lies with the production of zoospores. Although zoospores have a limited capacity for dispersal unless transported in moving water (Wilkinson *et al.*, 1981), they have a major significance in their role as precise "homing" agents, as their motility is linked to receptor functions for detection of environmental stimuli (Deacon & Donaldson, 1993). As homing responses are central to an understanding of zoosporic fungi and thus to attempts to exploit or control them, so may an understanding of possible chemotactic responses of AMF be important in their manipulation for the benefit of agricultural systems.

It is widely recognised that zoospore attraction is induced by chemical compounds present in root exudates (Hickman, 1970). Sites of maximum zoospore accumulation on roots occur at specific sites, typically coinciding with sites of maximum exudation ie. elongation zones and wounds (Zentmyer, 1961), implicating exudates as a major contributory factor in the response of zoospores to roots (Pearson & Parkinson, 1961). In general, taxis to culture-grown roots is not host specific, indicating a probable response by fungi to common components of root diffusates (Deacon & Donaldson, 1993). Amino acid fractions are often the most attractive (Chang-Ho & Hickman, 1970), but it is likely that taxis *in vivo* will involve amino acid combinations rather than specific individual molecules (Deacon & Donaldson, 1993). Zoospores of *Phytophthora* species have been observed to show a positive chemotactic response to a number of amino acids, while flavones and

isoflavonoids were less effective (Morris & Ward, 1992). However, as is always the case in natural systems, chemotactic responses are unlikely to be restricted to single-factor causes. Volatile compounds are also likely to be implicated, particularly in zoospore activity, as they are associated with roots in water-logged conditions which are also favourable for zoospore activity and infection (Duniway, 1983). Such conditions also cause a general increase in root exudation (Rovira, 1969).

Chemotaxis of the zoospores of *P. megasperma* Drechsler to primary roots of alfalfa seedlings *in vitro* correlates closely to susceptibility and resistance ratings obtained in varietal screening studies (Chi & Sabo, 1978). Chemotaxis and chemotropism are likely to be vital phenomena in the pathogenesis of root invading fungi in the genus *Phytophthora*, and possibly also other soil-borne pathogens which produce motile zoospores (Chi & Sabo, 1978). However, compounds which cause chemotaxis (attraction to a source) do not necessarily also result in chemotropism (growth up a concentration gradient) (Carlile & Tew, 1988), and in fact chemotropism to nutrients is rare in the higher fungi (Gooday, 1975). Any existence of any specific mechanism would confer obvious advantages over random contact for location of potential host plants.

Environmental responses occur throughout natural systems, and are observed and well documented in species other than fungi. The pattern of spread of many plant species is analogous to search pathways of foraging animals (Grime, 1979). As animals move to exploit fresh resources, so plants grow to use unexploited nutrients, water or light. There has been relatively little work carried out on the adaptive significance of plant morphology, although theoretical methods can be used to predict actual morphology. "Optimisation models" assess plant growth patterns, and are particularly feasible with clonal growth forms such as stolons and rhizomes where most growth patterns can be interpreted in terms of vegetative growth (Sutherland, 1990). Ramets of *Ranunculus repens* L. show contrasting growth dynamics according to habitat. Thus, in late succession a tightly packed "advancing front" of ramets expands slowly to form large clumps which are successful in a close, competitive environment. In contrast, early successional growth consists of widely spaced ramets which are able to explore a wide area and thus discover and occupy gaps in a sward (Lovett Doust, 1981).

Plant growth often follows a regular pattern, differences dictated by differences in "growth rules". Striking morphological differences may be largely attributed to simple modifications which occur as a result of adjustments to growth rules triggered by environmental stresses or stimuli. Typical morphology can be likened to "strategy", while foraging tactics represent deviations from strategy due to

an environmentally-mediated response. Models consider how modifications to the growth pattern can increase the ability of an individual to invest in more favourable areas. One source of accumulation at better sites is if branching is dependent on habitat quality. In stoloniferous plants, for example, growth in poorer patches is in a straight line and proliferation occurs on reaching the better source. Whatever criteria are used for habitat quality, branching is more frequent in good than in poor habitats. Branching angle appears unresponsive to environmental factors. Alternatively, plants may produce shorter internodes in good patches, thus increasing the chance of the next plantlet growing in the same patch. Such responses, which are apparently consistent within, and often between, species, allow plants to keep track of spatial changes in their environment through their foraging behaviour (Sutherland, 1990). A second possible foraging response of plants to their habitat will occur if they are able to respond to better sites in the same way as they respond to light, by growing towards the source. Rhizomes are capable of detecting nutrient gradients in soil and establishing roots in locations with higher concentrations.

As physiology can explain intra-plant movement of compounds, such as re-distribution of carbohydrates, so can basic morphological differences be explained in terms of physiology. A simple consequence of apical dominance is that a plant persists in uni-directional growth until conditions deteriorate and the apical bud dies. Once released from apical dominance, side buds will develop, allowing growth in a new direction. Apical dominance may also allow plants to exploit patches in another way. In good conditions, the suppressive effect of the apical bud disappears, thus allowing branching to occur.

This type of foraging behaviour is also applicable to other types of nutrient absorbing organs such as plant root systems and fungal mycelia. The root systems of different species are variable in their ability to proliferate in localised patches of high nutrient availability. Root systems of slower growing species may respond more readily to heterogeneous nutrient concentrations, showing greater relative allocation to higher nutrient sites (Crabtree & Bernston, 1994). Measurements of root architectural responses of *Betula lenta* L. to spatially heterogeneous ammonium and nitrate indicated that the most dominant influence was heterogeneity of form of nitrogen across a single root system, not the form of nitrogen *per se*. For *Betula lenta* foraging in regions of both nitrate and ammonium, it appeared that the plant reacted to perceived heterogeneity of nutrient supply as to a lower nutrient environment, and there was no striking difference between root architectures in nitrate and ammonium patches (Crabtree & Bernston, 1994). Thus, local root growth was not influenced by localised changes in nitrogen form. It was suggested that this

may occur as the result of a general root architecture in this species tuned to the exploitation of ammonium, which is the predominant nitrogen form in its natural habitiat range, that was not varied for nitrate (Crabtree & Bernston, 1994).

Whether nutrient absorbing organs are distributed at random through the soil volume or are selectively concentrated at nutrient rich microsites may be of considerable significance in terms of whole plant nutrient acquisition (St. John *et al.*, 1983b). Associations between hyphae and organic particles may come about through a dynamic process and need not result from coincidental or passive mechanisms such as negative geotropism. Both roots of forest trees and hyphae of AMF, which are principle nutrient absorbing organs, have been observed to associate with decomposing organic matter and other localised sites of nutrient availability (Kimmins & Hawkes, 1978; St. John *et al.*, 1983b) and to show enhanced proliferation in nutrient rich zones (St. John, 1983). However, although nutrient absorbing organs apparently associate physically in this way with decomposing organic material, which could imply a tropism or active hyphal orientation, the mechanisms by which such associations arise are generally inferred from indirect evidence. Since tropisms are known to occur in tip growing cells which are able to orientate their growth direction in relation to topographical changes in substratum (Sherwood *et al.*, 1992) and which can also exhibit chemotropism (Musgrove *et al.*, 1977), there has been increased interest over the past decade in testing the hypothesis that nutrient absorbing organs associate preferentially with organic materials through some locational mechanism (St. John *et al.*, 1983a).

However, results have led to rejection of the hypothesis that growing root or AMF hyphal apices respond to environmental cues in this way. Hyphae apparently do not encounter areas of high organic matter content with any greater frequency than that with which they encounter control areas (St. John *et al.*, 1983b) suggesting that random growth will ultimately result in microsite location and that proliferation of branching will occur after such an encounter (St. John & Coleman, 1983). This results in advantageous physical placement by allocating the maximum absorbing length to the richest microsites in the heterogeneous soil matrix. Such associations with rich microsites place roots or hyphae within a richer medium than the bulk soil and highly branched hyphae associated in this way with decomposing organic matter should confer advantages in ion uptake when this is limited either by diffusion rate or by competition with other organisms. Diffusion may often be limiting to nutrient uptake in soil, therefore the location of nutrient absorbing organs within a rich site can in many circumstances facilitate increased uptake (Barley, 1970) as the mean distance over which ions must diffuse from source to sink is less than if the mycelium is

distributed in other ways. If hyphae are in fact tropically attracted to nutrient rich microsites and are not randomly dispersed, then any passing through the relatively poor soil matrix are essentially acting as connections between those that have encountered such microsites. The fraction of connecting absorptive organs (whether roots or hyphae) is minimised, and the fraction actively absorbing maximised, when the most absorbing length is spatially allocated in this way (St. John *et al.*, 1983b). Thus less absorbing length is wasted at unproductive soil microsites than would occur if hyphal growth was truly random (St. John & Coleman, 1983). Although it is recognised that the development of the soil hyphal network does play a major role in determining the capacity of AMF to enhance growth, the determination of hyphal length, and particularly distribution, remains difficult. Many techniques used to monitor extra-matrical hyphae, which could potentially provide information on the soil variables responsible for the prevention or stimulation of growth, are destructive and are thus ineffective in analysis of spatial distribution.

The assumption that contact between hyphae and host plant is random seems paradoxical considering the obligate nature of the symbiosis and the relatively low density and mobility of AMF propagules in the soil (Gemma & Koske, 1988). Plant roots may be able to attract AMF via chemical messenger(s) which function prior to the establishment of infection (Koske, 1982). More recent evidence that shows stimulation of hyphal growth in *Gigaspora margarita* by the flavenol group of flavenoids, particularly in a CO_2 enriched atmosphere, lends support to claims that root exudates can influence hyphal growth (Chabot *et al.*, 1992). Germ tubes are apparently similarly attracted to roots via volatile organic compounds, which provide a more efficient medium of communication than water soluble compounds (Gemma & Koske, 1988). That hyphae are able to respond to chemical influences such as water soluble exudates from both root and other plant components has been shown by the apparent role of these in increased hyphal growth and branching (Carr *et al.*, 1985). If there is a communication system mediated by volatile messengers between members of the symbiosis in the pre-infection phase which is able to direct growth of germ tubes towards roots and roots towards spores, it seems possible that hyphae may similarly be able to respond to nutrient rich microsites.

3.2.3 Plant and Soil Influences on AMF

Events that condition whether or not a mycorrhizal association will form occur not only within the root tissue, at the rhizoplane and in the rhizosphere, but also at a wider level in the bulk soil. The rhizosphere represents the initial zone of influence of the root on the fungus, notably via root exudation. Its nutritional importance is not

restricted to AMF, but also influences numerous other soil microorganisms (Anderson, 1992). Plants clearly influence the type and extent of mycorrhizal formation, and plant species can greatly influence the distribution of AMF both in spatial and specific terms. Many of the plant-related impacts on AMF are mediated by the influence of their rhizosphere on the soil microbial population. Other plant related aspects of mycorrhizal development and function have been covered in depth in Section 3.2.1.

Soils commonly contain more than one species of AMF, the development of which is thought to vary with both soil type and depth. In addition, levels of soil extractable phosphate and organic carbon are known to play an important role (Abbott & Robson, 1991). However, relationships between levels of colonisation and soil chemical and physical properties appear markedly variable and, in contrast to plant effects on AMF, knowledge of the effects of the physical, chemical and biological properties of soils, particularly on hyphal form and function, is limited. Specific differences are likely to account largely for variability in responses of AMF to soil characteristics, but further information is required to clarify the role of AMF across diverse ecosystems (Abbott *et al.*, 1992). The major factor underlying the lack of information that is currently available is the difficulty associated with studying hyphae of AMF *in situ*.

Rudimentary definitions of soil, which relate fertility only to crop production potential (Russel, 1973), over simplify the complex nature of the soil-plant system. In fact, soil ecology, fertility and nutrient cycling are all closely interlinked, and combine to make the soil more than just an inert matrix for the accommodation of plant root systems. Thus, the soil is a key natural resource, and a critical ecosystem component. Its natural state and inherent fertility are determined by the capture of inputs from atmospheric deposition, and through weathering etc (Miller & Jastrow, 1992). Its intrinsic quality is determined by its chemical, physical and biological properties and their interactions, as modified by soil management practices. Ecosystem disturbance can significantly affect AMF population structure as a result of changes to the physical, chemical or biological status of the soil, which can directly or indirectly affect AMF. Alternatively the effects may be mediated through changes in the species composition of the vegetation and thus the balance of host to non-host plants, which will have knock-on effects on AMF population density and structure (Abbott & Robson, 1991). Soil disturbance which results from agricultural practices is a primary cause of shifts in AMF populations. These changes can occur because of, for example, erosion, cultivation practices, fertiliser addition, rotational and cropping practices. Soil management can have a major impact on AMF, through the application of

pesticides and fertilisers, removal of topsoil which contains the majority of viable AMF propagules, cultivation techniques which disrupt the external mycelium, or implementation of practices that change the balance between mycorrhizal and non-mycorrhizal species (Rhodes, 1980). Changes in soil fertility due to amendments with either mineral fertilisers or organic matter can markedly influence root infection and spore production in AMF. There is considerable evidence for negative impacts of applied mineral fertilisers, and some for the positive influence of organic matter (Hayman, 1982), most notably as a result of promotion of saprophytic growth of AMF in the soil (Hepper & Warner, 1983; Warner, 1984). The ability of hyphae to respond to such microsite differences in the soil (eg. St. John *et al.*, 1983a & b) is probably the underlying mechanism behind the creation of the hyphal network. However, although specific effects of soil fertility have been demonstrated at specific sites, it is less easy to find consistent relationships between sites (Hayman, 1982).

Compaction of soil by agricultural machinery decreases the pore size distribution and thus restricts pathways of air, water and nutrient movement, as well as causing mechanical impedance of root growth. This in turn reduces the soil volume explored by the root system, and the uptake of relatively immobile nutrients such as phosphorus (Nadian *et al.*, 1997). Whether changes in root growth in compacted soils affect colonisation by AMF, and whether there are any direct effects on the growth of the extra-radical mycelium is not yet known (Nadian *et al.*, 1997). Growth and development of AMF are affected by many environmental factors, and soil suitability for the growth of AMF hyphae will vary (Abbott & Robson, 1985). The best documented of these factors is undoubtedly soil phosphorus supply, which is negatively correlated with mycorrhizal growth response and with the extent of fungal colonisation (Abbott *et al.*, 1984). Soil compaction has been shown to result in an increased uptake of phosphorus per unit length of root, indicating that mycorrhizal uptake efficiency increases in compacted soil. This may most obviously be attributed to AM fungal "indifference" to either compaction or changes in root growth which arise from this, indicating that changes in root morphology induced by compaction may compensate in terms of AMF colonisation through the increased root volume for colonisation per unit length of root (Nadian *et al.*, 1997). However, mycorrhizal growth response decreases as soil compaction increases, an observation attributed to the concomitant reduction in root length and thus in arbuscular root length, which considerably decreases the surface area available for phosphorus uptake and transfer. Established colonisation within the root appears to be buffered against the decrease in oxygen concentration associated with compacted soil by the root cortex. The external

hyphae are more likely to be sensitive to reduced aeration because they are in direct contact with the soil atmosphere (Nadian *et al.*, 1997).

Difficulties in differentiating between AMF and other fungal hyphae in the soil make it difficult to assess the effects of compaction on the external phase of the symbiosis. It is likely that physical alteration in compacted soil will displace hyphae of larger diameter, such as the runner hyphae described in section 1.5.2. and that, assuming a relationship between total root length and length of external hyphae, soil compaction will also influence extra-radical hyphae indirectly through a reduction in root length (Nadian *et al.*, 1997). As species of AMF have been shown to differ in the length of external hyphae produced per unit length of infected root (Abbott & Robson, 1985), plant growth effects arising from changes in root length that occur as a result of compaction are thus likely to be mediated by the AMF population structure.

Observed immediate effects of soil disturbance on colonisation by AMF have been attributed to disruption of the external mycelium, which constitutes an important component of the inoculum potential of an undisturbed soil (Evans & Miller, 1990). Substantial reductions in absorption of phosphorus and zinc resulting from the disruption of a pre-existing mycelial network have also been associated with soil disturbance. It appears likely that such effects occur because of disruption of the mycelium *per se* rather than because of the removal of connections between it and older infected root systems (Evans & Miller, 1990). Maintaining the integrity of the external hyphal network is critical, as this phase of the AMF reflects the efficacy of the symbiosis as a whole. Not only is the soil important to the development of the hyphae, but the hyphae are also important in aspects of soil stability as a result of aggregation of soil particles. Little is known about the precise mechanisms by which formation and stabilisation of macroaggregates occur, or which species of AMF are most efficient at soil stabilisation. However, it is thought that aggregate stability is related primarily to external hyphal length (Tisdall, 1991).

Both chemical and physical attributes of soil have been the subject of much research emphasis, but biological aspects are more difficult to define. Soils are characterised by an inherent biomass, which is bolstered by the production of a transient biomass in association with plant roots or crop residues. Depending on the particular soil, plant and microbial populations, rhizosphere activity can stabilize or de-stabilize the soil (Lynch, 1984). The physical and chemical properties of the soil are modified by dynamic biological processes, many of which are bi-directional. Thus, the soil contains a complex and highly integrated community of microorganisms which both influences, and is in part determined by, the physico-chemical parameters of the

soil (Kennedy & Smith, 1995). The effects of soil on AMF populations and functioning are largely influenced by the soil microbial population, which is an essential element of a healthy soil. Equally, the AMF itself can modify both physical properties of the soil and its microbial associates. That these microbes may have positive or negative effects on development of the AMF is indicative of the self-regulatory nature of the system. The hyphal network formed by AMF in soils is a key component in nutrient cycling in natural ecosystems, and is a major sink for both carbon and other elements. Its role in nutrient dynamics is critical in ecosystem sustainability, allowing the cycling of nutrients between different components of the biomass (Jeffries & Dodd, 1996).

In the vicinity of roots, the soil is profoundly influenced by both root-derived nutrients and microbes, as the rhizosphere is a zone of tremendous biological activity. Its characteristics differ from those of the bulk soil in increased respiration rates, higher content of low molecular weight organic compounds, lower concentrations of oxygen and nutrient ions, lower water availability and altered pH. In contrast to the general properties of the bulk soil, which provides a harsh environment for microbial populations, the rhizosphere stimulates their activity and development. The rich carbon sources of the rhizosphere are utilized by the soil bacteria, increasing available energy supplies and thus further promoting microbial colonisation (Vosátka, 1996). AMF significantly influence the rhizosphere microflora by inducing changes in root physiology and exudation, and through the provision of physical or nutrient substrates; conversely, microbial associates of AMF may profoundly affect the further development of the hyphae in the soil, for example through the absorption and translocation of microbial metabolites. Although the host plant provides the primary energy source for hyphal development, the extent of the hyphal network is strongly influenced by the soil microflora (Linderman, 1992). Soil microbial populations act as important mediators in the plant-fungal interaction, controlling decomposition and nutrient availability to both partners in the symbiosis (Vosátka, 1996).

The presence of AMF can modify soil microbial populations either directly or indirectly, notably because of the changes that occur at the root-soil interface as a result of the development of the extra-radical hyphae. Specific partner bacteria appear able to improve growth of AMF in both the symbiotic and non-symbiotic state, representing carbon-rich microsites which are favourable for both symbionts and may attract hyphae, subsequently supporting their growth and metabolic activity (Vosátka, 1996). Bacterial inoculum is thought primarily to influence extra-radical hyphae of AMF, and has been found to stimulate both their total and active length. That heat killed bacteria also stimulate hyphal growth indicates the potential of bacterial

populations as a source of microbial organic matter (Gryndler & Vosátka, 1996). Conversely streptomycetes, amongst the most common of the soil actinomycetes, commonly have antibiotic producing capabilities against a range of microorganisms including fungi (Ames, 1987). Members of the microbial population may also be hyphal feeders which can disrupt the integrity of the extra-radical hyphal system (Sylvia & Williams, 1992). Whether fungal-microbe interactions are in general beneficial, harmful or neutral to the efficacy of AMF has yet to be determined (Ames, 1987).

Evidence suggests that considerable functional diversity exists within the AMF both within and across habitats, indicating that changes in mycorrhizal populations may significantly influence resource acquisition and plant growth (Allen *et al.*, 1995). Fungi are individual organisms which have their own relationships with climate and soils, and specific environmental requirements may exert a more powerful influence than specific host needs. The functioning of ecosystems is governed largely by soil microbial dynamics, and therefore differences in microbial properties and activities of soils are important (Kennedy & Smith, 1995). Relationships between microbial diversity, soil functioning, plant health and ecosystem sustainability are seldom quantified.

3.3 General Methods

3.3.1 Mycorrhizal Stock Cultures

Stock cultures of mycorrhizal plants were established by inoculation of seedlings with roots colonised by specific mycorrhizal fungi. Roots were assessed for mycorrhizal colonisation by observation under a binocular microscope (Wild M10, Leica, UK) after selective staining using the method of Koske & Gemma (1989; Section 3.3.2). Levels of infection sufficient to allow multiplication of cultures were usually achieved in approximately five weeks. Multiplication was carried out using the method described below.

Root inoculum was chopped into small pieces and mixed thoroughly at a ratio of 1:6 with sterilised potting medium (Seed & Cutting Compost, John Innes, UK) in which the new seedlings were to be established. The compost was sterilised by autoclaving twice for 60 minutes each time at 121°C and 15 psi (Midas 32, PriorClave, UK). Seeds were surface sterilised in 10% (v/v) Domestos (Lever Ltd., UK) and rinsed thoroughly in sterile water. All pots and seed trays used for mycorrhizal stock plants were pre-sterilised in 70% (v/v) methanol and rinsed thoroughly in sterile water. In addition, extreme care was used in setting up cultures to prevent cross-contamination between species. For this reason, pots containing plants colonised by specific mycorrhizal fungi were also kept in separate trays.

Cucumber was used as the preferred stock plant in the short to medium term because of its ability to produce abundant white roots in which mycorrhizal structures are easily visible without staining. For longer term maintenance of stocks, species with greater longevity such as ribwort plantain (*Plantago lanceolata*), strawberry (*Fragaria* x *ananassa* Duchesne), sweetcorn (*Zea mays*), leek (*Allium porrum* L.) and onion (*Allium cepa* L.) were used. The stock plants were maintained in a glasshouse, and watered as necessary so that they were kept moist but not allowed to become too wet. Long term stock plants were watered weekly with one-third strength Hoagland's nutrient solution (Appendix 1).

3.3.2 Mycorrhizal Staining (Koske & Gemma, 1989)

Harvested roots were washed thoroughly under running water to remove soil residues and heated in 2.5% (w/v) potassium hydroxide for approximately 10 minutes (according to host plant species) at 90°C, then bleached in alkaline hydrogen peroxide for 15 minutes. Roots were rinsed thoroughly in tap water after each stage. They were then soaked in 1% (v/v) hydrochloric acid for 30 minutes, and stained in acidic glycerol containing 0.5% (w/v) trypan blue for approximately 15 minutes at 90°C.

A modified staining technique suitable for rapid assessment of colonisation was later introduced in which roots were heated in 2.5% potassium hydroxide for approximately 10 minutes and then stained in trypan blue by microwaving at high power for 20-30 seconds (M. Munro, pers. comm.). This method was used to check the progression of infection where accurate quantification of mycorrhizal colonisation was not necessary.

3.3.3 The Use of Root Pieces as Inoculum

All work was carried out using mycorrhizal root pieces as inoculum. Regrowth hyphae from root pieces are likely to give a more accurate representation of the response to stimuli exhibited by extra-radical mycelium than are germ tube hyphae obtained from spores, which have a different role in the mycorrhizal symbiosis. In addition, extraction procedures for obtaining mycorrhizal spores from soil can be unreliable, and there are low numbers of spores in young cultures. The species used was *Glomus etunicatum* (S329) obtained from the International Collection of Arbuscular and Vesicular-arbuscular Mycorrhizal Fungi (INVAM, USA), unless otherwise stated.

Roots were collected from mycorrhizal pot cultures (Section 3.3.1) and washed under running water. Unstained roots were examined microscopically and root sections containing large numbers of vesicles, clearly visible in unstained roots, were identified. Selected roots were cut into transverse sections of approximately 1-1.5mm in length.

3.3.4 Visualisation of Unstained Hyphae

3.3.4.1 Photographic Enlargement

Petri dishes containing germinating mycorrhizal hyphae on membranes (Section 3.4) were placed into a large format photographic enlarger (Varicon de Vere) and projected directly onto positive lithographic film (K. Ritz, pers. comm.) for a 20 second exposure time. The film was developed for 5 minutes (Bromophen paper developer, Ilford, UK) and fixed for 1-2 minutes (Hypam fixer, Ilford, UK). The lithographic images were subsequently enlarged.

3.3.4.2 Microscopic Visualisation

Video-linking a binocular microscope (Wild M10, Leica, UK) to a monitor via a low light camera (KMI KCD-1CU) resulted in capture of images on-screen which could be traced manually onto acetate sheets. At low power, this method gave a final field of view on-screen equivalent to 8 x 6 mm. An alternative method which enabled

a larger field of view to be visualised was subsequently used, in which the microscope was linked directly to an image analysis system (Quantimet 600, Leica, UK) via the red channel of a 3CCD colour video camera (Sony, Japan), giving a final low power field of view equivalent to approximately 15 x 12 mm. Images were captured and stored on disc as .tif files for subsequent assessment. This method was adopted for hyphal growth studies as it allowed acquisition of accurate images which recorded mycelial development in real time. Image editing by grey level processing (Section 3.3.4.3) improved contrast, but detection processes required prior to measurement remained difficult because of low contrast in the image and background "noise". Print-outs of images acquired in this way (Plate 2a) were therefore traced manually onto acetate sheets to provide high-contrast maps of mycelial growth patterns (Plate 2b). These were viewed via a macro-viewer and digitised for further analysis.

3.3.4.3 Image Processing and Analysis

Image preparation and analysis of mycelial maps was carried out using a Quantimet 600 (Q600) Image Analysis System (Leica Ltd., UK) controlled by Qwin software (Leica Ltd., UK) which runs under Microsoft Windows.

Initial images were obtained by directly video linking a binocular microscope to the image analysis system (Section 3.3.4.2). Optimum images were obtained by varying the basal illumination of the microscope between light and dark field, and stored on disc as .tif files (Section 3.3.4.2). Stored images were recalled and processed prior to image analysis, allowing detail to be picked out and unwanted artefacts to be removed. Image processing was required to enhance the contrast between the hyphae and the dialysis membrane used as substrate, and to remove background "noise". The original image was stored as image 0. After each processing operation, successive images were stored independently (as image 1, 2 etc.). This maintained each image separately, allowing the result of any given processing operation to be recalled. The original image was always available for reference in image store 0. The images were amended using a combination of the following processes:

Look-up table (LUT) transforms. These are used in image acquisition and processing to perform tasks relating to analogue-to-digital conversions, image arithmetic etc., and enable remapping of image grey levels. LUT transforms were used to invert the image (ie. grey level 0 is converted to 255, 1 to 254, 2 to 253 etc.) where this improved contrast between hyphae and background.

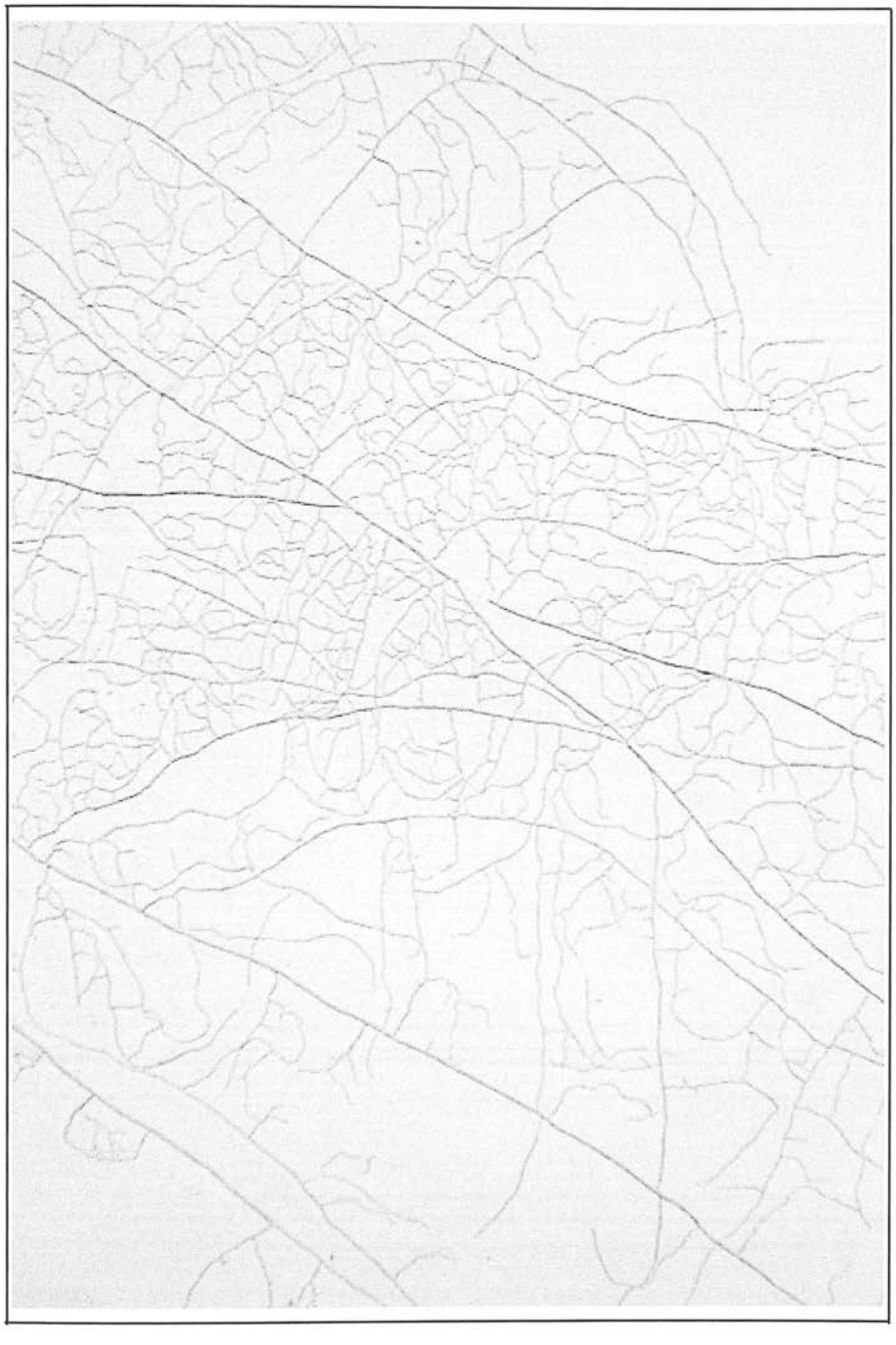


Plate 2a: Extra-radical hyphae of AMF growing on dialysis membrane (Wild M10, Leica, UK)

Plate 2b: Photo-reduction of a manual tracing of an AM fungal mycelium
(blank area in centre indicates point of inoculum)

Image amendment - linear closing. Closing with linear structuring elements was used to create a filter sensitive to elongated features. The linear closing operation works on the background of the image, retaining portions elongated in one or more of the closed directions while removing non-elongated regions. The use of this operation enabled removal of debris etc. from the membrane, thus clarifying the image for subsequent processing.

Image transform - black sharpen. This transformation is also useful for enhancing features of interest and removing unwanted features, and was used to emphasise black detail.

Convolute - sharpen. Convolutions were used to create a sharpening effect where necessary. This created a defined image, but with background "noise" which limited its use as a pre-detection operation. Because of this, print-outs of the processed image were used as a basis for manual tracings on acetate sheets, which provided clear mycelial maps (Figure 3.2).

Image analysis was carried out on these mycelial maps. Image analysis operates by generating a signal proportional to the intensity of illumination, which is then digitised into picture elements (pixels). The image brightness is sampled at each pixel, and the Q600 then analyses the digital representation of the image. The processed image is converted to a binary image by thresholding, and measurements are taken.

The following steps were used:

Calibration: The system was calibrated using a graticule imaged at the same magnification as the fungal mycelium. Print-outs of calibration images were placed on a light table under a zoom lens. Q600 calibration is carried out by measurement against a known distance, in this case the imaged graticule, using the mouse to indicate a rectangle stretched between two fixed points on the scale This distance was measured in pixels, and the actual size entered in absolute units (mm). The Q600 calculated the necessary calibration value.

Image capture: The mycelial map was placed on a light table under a zoom lens, and the image captured into the Q600 image store (image 0). For images which showed a large or complex mycelial spread, it was sometimes necessary to split the map into sub-sections and analyse these individually.

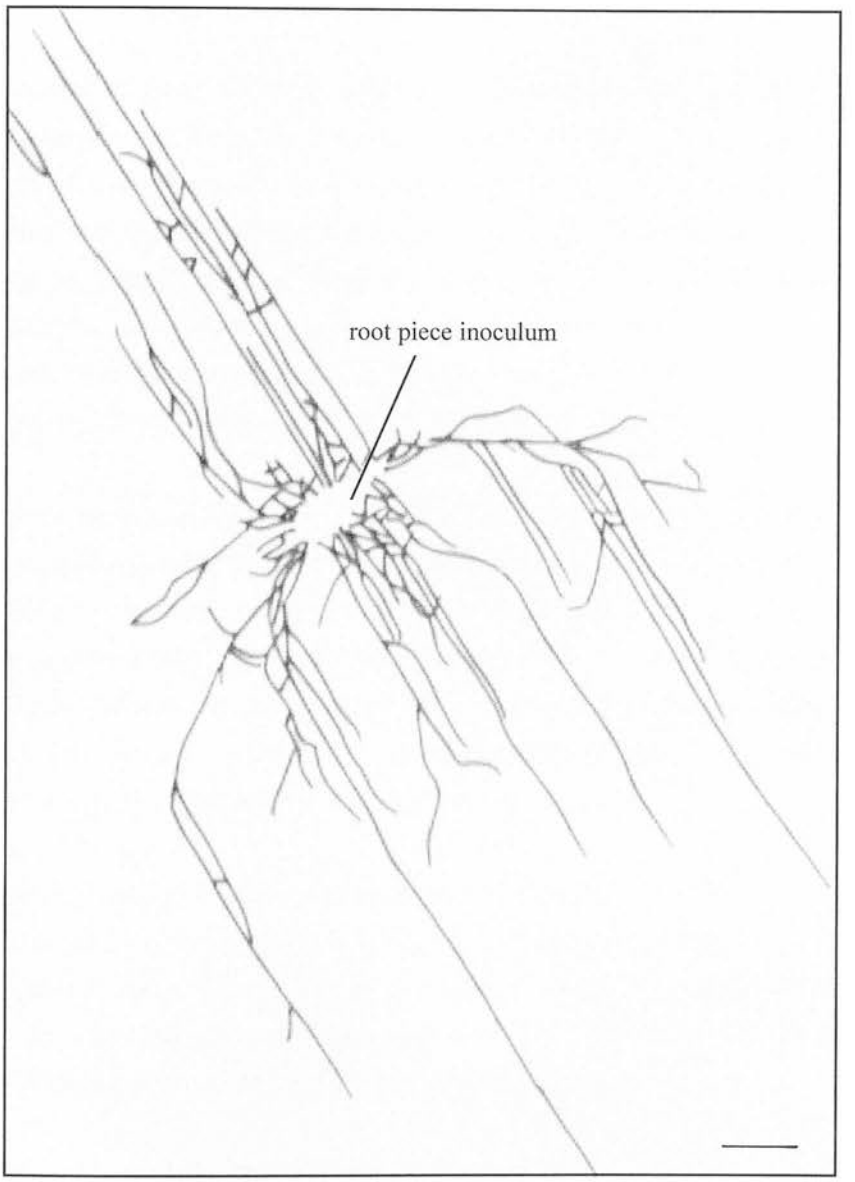


Figure 3.2: Example of manual tracing of AMF mycelium
(Scale bar represents 1mm)

Detection: Following image acquisition the image was detected by thresholding. Pixels darker than the selected threshold level were identified and displayed as a blue image. The blue display was matched to the processed image (in this case the mycelial map). All the pixels which met the defined threshold were selected and transferred to create a binary image (Binary 0), which identified the region of interest and defined the boundary of the object to be measured.

Binary amendment: False detail in images produced by detection was removed by binary image processing. Because operator eye is able to discriminate between wanted and unwanted detail where the image analyser itself is not, modification was carried out by manual image editing using the binary edit mode. This allowed regions of the binary image to be amended manually by erasing or by completion of imperfectly detected features. An "ultimate skeleton" operation was then used to reduce the image to lines of single pixel thickness. This process also reduced connected regions to a single pixel. The amended image was stored as binary image 1.

Measurement: Measurements which described the properties of the complete field of view (field measures) were used. Field data sums the values of all the objects in the measuring frame, regardless of whether they are touching or separate. Field perimeter measurements were taken on binary 1 images, and results were divided by two to give mycelial length. Actual length measurements are not appropriate in image analysis, because length is taken as distance between furthest apart points of an object rather than total length of the summed objects themselves.

Statistical analysis of data was carried out using the Minitab for Windows statistics package (Version 10; Minitab Inc., USA). Because of the variability of the data, which meant that it did not fall within a normal distribution, non-parametric tests were used for statistical analysis. For group data, the Kruskal-Wallis test was used. The Mann-Whitney test was used in the comparison of pairs.

3.4 Development of Experimental Methodologies

Hyphal growth from root pieces (Section 3.3.3) colonised by the mycorrhizal fungus *Glomus etunicatum* was tested on dialysis membrane (Medicell International Ltd., UK) and cellulose nitrate filters (Whatman Paper Ltd., UK) in systems which restricted hyphae to a 2-D growth form. As moisture retention is important in preventing hyphal desiccation, and in maintenance of a water film around the hyphae

which generates contrast necessary for hyphal visualisation, a number of different methods of retaining moisture were tested (Table 3.1).

Table 3.1 Summary of methods used to retain moisture in experimental systems for hyphal regrowth from mycorrhizal root pieces

Substrate for inoculation	Overlay	Additional moisture source	Experimental duration (d)
Capillary matting[1] + dialysis membrane[2]	Cellulose nitrate filter[3]	Capillary matting	42
Cellulose nitrate filter	Cellulose nitrate filter	Capillary matting	7/14
Phytagel[4] + dialysis membrane	Cellulose nitrate filter	Capillary matting	42
Washed gravel + dialysis membrane	Cellulose nitrate filter	Capillary matting	42
Washed gravel +cellulose nitrate filter + dialysis membrane	Cellulose nitrate filter	Capillary matting	42

1: Netlon, UK

2: Medicell International Ltd., UK

3: Whatman Paper Ltd., UK

4: Sigma Chemical Co., USA; 1.5 gl^{-1} working strength

The experimental units used were 10 x10 cm disposable Petri dishes (Bibby Sterilin Ltd., UK). Membranes and filters were cut to size; membranes were boiled twice for 30 minutes each time in deionised water to remove plasticisers before use.

Dishes were sealed with Nesco film (Bando Chemical Ind., Japan) and incubated in the dark at 25°C. Apart from those incubated between cellulose nitrate filters which were visualised by destructive staining with cold trypan blue after 7 days, germinating hyphae were observed non-destructively over a number of weeks using a binocular microscope. The experiment was terminated after 6 weeks (t=42 d) after which visual assessments of hyphal growth were made. Control experiments were run simultaneously using both the cellulose nitrate filter "sandwich" technique, and root pieces inoculated onto capillary matting/dialysis membrane with no overlay.

At the end of the experiment, root pieces showing extensive hyphal growth were retained to test the infective capacity of hyphae *in vitro*. Cucumber seeds (Bush Champion, Hydra/Chase, The Organic Gardeners, UK) were surface sterilised for 10 minutes in 10% (v/v) Domestos (Lever Ltd., UK), rinsed thoroughly in sterile water and pre-germinated on Phytagel. The seedlings were transferred to the dish containing the *in vitro* hyphae, and the root systems were covered over with a cellulose nitrate filter to prevent desiccation and maintain darkness. Mycorrhizal infection was assessed after 21 days by destructive staining with trypan blue.

Following these initial attempts to produce and visualise hyphae, a number of further techniques were tested:

Mycorrhizal root pieces were inoculated onto dialysis membrane in Petri dishes, and overlaid with a second membrane. The membranes were kept moist using capillary matting and the dishes were sealed with Nesco film and incubated in the dark at 25°C. Initially, observations of hyphal growth between the two membranes were carried out *in situ* using binocular microscopy. Subsequently the upper membrane was replaced with a cellulose nitrate filter as it appeared that the use of the two membrane technique was affecting the relationship between moisture and resolution, and thus compromising hyphal visualisation. Observations were carried out over a 5 week period.

Mycorrhizal root pieces were inoculated onto a membrane between two moistened cellulose nitrate filters (5 x 5 cm) and sealed into Petri dishes using Nesco film. No additional moisture source was used. The dishes were incubated in the dark at 25°C and hyphal development was monitored over a 4 week period.

Mycorrhizal root pieces were inoculated onto single membranes with damp cotton wool pads as a moisture source. The dishes were sealed with Nesco film and incubated in the dark at 25°C. Hyphal development was monitored over a 3 week period. This method was adopted for further work, as *in-situ* non-destructive hyphal observations could be carried out. Where hyphal growth exceeded the field of view, overlapping images were taken and the whole mycelium was reconstructed by editing of the print-outs.

3.5 Experimental Procedures

3.5.1 Control Experiments

Control experiments were run using single mycorrhizal root pieces (Section 3.3.3) inoculated centrally onto 5 x 5 cm squares of dialysis membrane (Medicell International Ltd., UK) in Petri dishes, and kept moist with cotton wool pads. The

dishes were sealed with Nesco film (Bando Chemical Ind., Japan) and incubated in the dark at 25°C (Section 3.4). The effect of root piece orientation was also tested by uni-directional placement of root pieces.

Further experiments were carried out using the same system with the addition of agarose blocks placed on the membrane as point stimuli. These were created using discs of 1.5% molecular grade agarose gel (Sigma Chemical Co., USA). Plugs were cut from the gel using a sterilised glass Pasteur pipette, and sectioned transversely to give discs of approximately 1mm height and diameter. In each experiment carried out using point sources of stimuli, two discs were placed diagonally opposite each other at distances of 5mm from the root piece. This distance was chosen as a result of observations from preliminary experiments (Section 3.4) which showed that hyphae were able to grow approximately 2.5mm in a given direction within 7 days, and 5mm within 21 days. Hyphal growth was observed microscopically on a daily basis, and images were saved onto disc as .tif files (Section 3.3.4.2). Measurement of hyphal growth parameters was carried out using the Quantimet 600 image analysis system (Leica, UK) (Section 3.3.4.3).

3.5.2 Effects of Natural and Synthetic Plant-Derived Test Compounds

In experiments requiring generation of a heterogeneous environment by the addition of placed stimuli containing plant-derived test compounds, point sources were created from agarose impregnated with the test compound.

3.5.2.1 Root Exudates and Plant Flavonoids

Seeds of test plants were surface sterilised using 10% Domestos (Lever Ltd., UK) for 10 minutes. Either cucumber or onion was used as the host plant species for exudate collection, depending on the host plant of origin of the root piece inocula used in a particular experiment. Oilseed rape was used as the non-host test plant. Seedlings were germinated on agarose for 14 days to allow full impregnation with root exudates. For experiments using plant flavonoids as test compounds, hesperetin (3', 5, 7-trihydroxy-4 methoxyflavanone; Sigma Chemical Co., USA) and naringenin (4', 5, 7-trihydroxyflavanone; Sigma Chemical Co., USA) were used at a concentration of 1.5μM (Gianinazzi-Pearson *et al.*, 1989). Discs were cut from the agarose medium containing the test compounds and placed on the dialysis membrane as point stimuli. The effect of adding the test compound both at the start of the experiment and after seven days hyphal growth were tested.

Following inconsistent results of early experiments aimed at showing directional growth responses to heterogeneous environments, the method was

modified to examine the general growth effects of test compounds in a homogeneous substrate. Plantlets were grown in agarose for up to 14 days and then carefully removed. The method assumed complete diffusion of plant derived compounds throughout the medium. The membrane was placed directly onto the gel and inoculated with the root piece. No additional moisture source was necessary. To ensure complete homogeneity of the substrate, low melting point agarose was later substituted so that following removal of the plantlets the gel could be re-melted, mixed thoroughly and allowed to reset before addition of the membranes and root pieces. The melting temperature of the agarose was sufficiently low to ensure that active compounds exuding from the roots were not denatured.

Exudates of colonised host plants were also tested. Cucumber seeds were surface sterilised in 10% (v/v) Domestos (Lever Ltd., UK) for 10 minutes and germinated on agarose gel. Plantlets were placed between cellulose nitrate filters with 20 mycorrhizal root pieces and transferred to sterile Magenta pots (Sigma Chemical Co., UK) containing sterile sand. These were maintained under a light bank for 3 weeks. Control and non-host plants used in the same experiments were similarly pre-germinated, placed between filters without mycorrhizal inoculum and transferred into Magenta pots. Once the host plants were colonised by the fungus, all plantlets were carefully removed from the pots and transferred into Petri dishes containing 20 mls low melting point agarose. After one week the plantlets were removed and the agarose melted and repoured. Membranes were then inoculated with single 1 mm mycorrhizal root pieces as previously described.

Hyphal development was imaged and measured after 7 and 14 days as described in sections 3.3.4.2 and 3.3.4.3.

3.5.2.2 Root Extracts

Seeds of test plants were surface sterilised using 10% Domestos (Lever Ltd., UK) for 10 minutes. Cucumber was used as the host plant species, and oilseed rape as the non-host. Seeds were germinated in Terragreen (Oil Dri (UK) Ltd.) in sterile Magenta vessels (Sigma Chemical Company, USA) and grown under a light bank (16 hour light; 8 hour dark) until the root systems were well developed. Where colonised host plant root extracts were used, pre-germinated cucumber seedlings were inoculated by sandwiching them between sterile cellulose nitrate filters (Whatman Paper Ltd., UK) with 1mm sections of AMF colonised root. The filter sandwiches were transferred into Magenta vessels containing Terragreen, and maintained under a light bank until colonisation occurred.

Plantlets were carefully removed from the rooting medium and the root systems were cut off and washed in sterile distilled water. Approximately 5 g of root were ground up in sterile distilled water using a pestle and mortar. The extract was filtered through 200μ mesh to remove plant debris and the volume was made up to 250 ml (or equivalent weight:volume) 1.5% low melting point agarose (BDH Ltd., UK). This was poured (20 mls per plate) into 90 mm triple vent Petri dishes (Bibby Sterilin Ltd, UK). Membranes (Medicell International Ltd., UK) were then placed on the agarose and inoculated with single 1mm mycorrhizal root pieces. Hyphal development was imaged and measured after 7 and 14 days as described in Sections 3.3.4.2 and 3.3.4.3.

3.5.2.3 Heat Stability of Root Exudates

Seeds of cucumber (host) and oilseed rape (non-host) were surface sterilised using 10% Domestos (Lever Ltd., UK) for 10 minutes, then transferred into pre-sterilised, covered boiling tubes containing paper cylinders (Horticultural Sorbarods, Ilacon Ltd., UK). The lower half of each tube was wrapped in foil to exclude light. The tubes were maintained under a light bank (16 hour light; 8 hour dark) until the root systems were well developed. This change of method was adopted to overcome the potential problem of unequal diffusion of exudates through the agarose gel used in previous experiments. Two Sorbarods from each treatment (host, non-host and control) were then placed in 200 ml sterile distilled water in covered conical flasks and shaken on an orbital shaker (Edmund Bühler, Germany) for two days at 200 rpm. Exudates were filtered through 200 μ mesh and made up to 1.5% low melting point agarose gel (BDH Ltd., UK). Membranes (Medicell International Ltd., UK) were placed on the agarose and inoculated with single 1mm mycorrhizal root pieces. Hyphal development was imaged and measured after 2 days as described in Sections 3.3.4.2 and 3.3.4.3.

3.5.3 Exudate Fractionation and AM Fungal Bioassays

Plant root exudates, obtained as described above (Section 3.5.2.3), were fractionated using Macrosep™ microconcentrators (Filtron Technology Corporation, USA) to obtain fractions of molecular sizes <3K, 3-10K and >10K.

The AM fungal bioassay was carried out using 50mm Petri dishes (Bibby Sterilin Ltd., UK) containing filter paper (No. 1, Whatman Paper Ltd., UK) moistened with sterile distilled water. Gridded cellulose nitrate membranes (Whatman Paper Ltd., UK) were placed over the damp filter papers, and inoculated with 1mm mycorrhizal root sections. The dishes were sealed with Nesco film (Bando Chemical

Industries, Japan) and incubated in the dark at 25°C for two days. Hyphal development was assessed visually using a scoring system based on the membrane grids to describe the extent of growth:

Table 3.2 Summary of scoring system used in fungal bioassays of fractionated root extracts and exudates

Score	Hyphal Growth
0	No hyphae
1	Hyphae cover < 4 grid squares
2	Hyphae cover 4 grid squares - growth sparse
3	Hyphae cover 4 grid squares - growth dense
4	Hyphae cover > 4 (<16) grid squares - sparse
5	Hyphae cover > 4 (<16) grid squares - dense
6	Hyphae cover > 16 grid squares

3.5.4 Exudate Analysis

Chemical analysis of plant root extracts and exudates was carried out using a number of techniques for separation of different compounds.

3.5.4.1 Protein and Amino Acid Analysis

Protein absorbance spectrophotometry of plant root extracts and exudates showed that all contained detectable quantities of protein. Following absorbance spectrophotometry, samples were run through a high performance liquid chromatograph (HPLC) for amino acid analysis. The method used was one sensitive to individual amino acids developed at the Scottish Crop Research Institute (SCRI, Invergowrie, Dundee) for amino acid analysis of transgenic potato tubers. O-phthaldialdehyde was used as a derivisation agent and resolution of amino acid derivatives was obtained with a two-solvent gradient using (A) sodium acetate buffer containing 4.5% 1-4 dioxane and 3% 2-propanol (HPLC grade), and (B) methanol containing 1.5% 1-4 dioxane and 1.5% 2-propanol.

20µl of sample was placed into a vial and loaded in to the autosampler. 80µl of O-phthaldialdehyde reagent was automatically mixed with this, and 20µl was injected onto an Adsorbosphere OPA HR 5U column (Alltech Associates Inc., USA). The gradient ran from 0-10% B over 15 minutes, 55% B over the following 25 minutes, and then increased to 100% B over a further 10 minutes. The column was

washed with 100% B for two minutes and re-equilibrated with 100% A for three minutes prior to injection of the next sample. Measurements were made at an excitation wavelength of 325 nm and emission wavelength of 465 nm using a Kontron fluorescence spectrophotometer. 20µl samples of 0.025µmol/ml standards were run for comparison.

A second series of analysis was carried out on host and non-host exudates using acid hydrolysis for amino acid separation by HPLC. Acid hydrolysis increases the free amino acids content of a sample, and is suitable for determination of all amino acids except methionine, cysteine and tryptophan. 6N hydrochloric acid was added to the samples and mixed in an ultrasonic bath for 15 minutes. Samples were then flushed with oxygen-free nitrogen for 1 minute and the sample tubes were sealed tightly prior to hydrolysis for 24 hours in a pre-heated heating block at 110±1°C. After hydrolysis, samples were cooled to room temperature. Amino acids in the hydrolysates were derivatised with O-phthaldialdehyde and 50µl subsamples were injected into the HPLC for separation on an Adsorbosphere OPA-HR column (Alltech Associates Inc., USA) using eluents as above. The gradient ran from 0-7% B over 7 minutes, 46% B over 33 minutes, 76% B over 40 minutes and back down to 0% B at 41 minutes. Measurements were made at an excitation wavelength of 325nm and emission wavelength of 465nm. Internal standards were run as sensitivity tests.

3.5.4.2 Carbohydrate Analysis

Carbohydrate analysis was carried out using an integrated high speed liquid chromatography system (DX500, Dionex, USA). This simplifies system configuration for any ion chromatography or HPLC application using a technique similar to standard HPLC, but with a detector based on conductivity rather than absorbance. The detector works by constant oxidisation of ions and measurement of the redox potential each time it oxidises, giving an increasing charge as a compound elutes and a decreasing charge as the peak tails off. The system consisted of an AS40 automated sampler and injector unit run by an air compressor, an ED40 electrochemical detector and GP40 gradient pump and a Dell computer with 'Peaknet' software. 25 µl samples of test compounds were injected onto Carbohydrate PA100 Guard and PA100 analytical columns, with helium pressurised solvents (100mM NaOH) using an isocratic gradient at 1ml/min. Detection was by pulsed amperometry, PAD-2 (gold electrode).

Following on from this, cation exchange was carried out on the non-host exudate and on colonised and non-colonised host root extracts using cation exchange columns (Alltech Associates Inc., USA). The columns contained styrene

divinylbenzene as the polymer, with a particle size range of 35-75μ, a molecular exclusion limit of 1000 Daltons and an exchange capacity of 2.1meq ml^{-1}. These samples were chosen because they contained a relatively large number of compounds when analysed for total carbohydrate. The columns were pre-rinsed with deionised water and the sample washed through under vacuum. Samples were then run through the Dionex.

3.5.5 Measurement of Hyphal Branching and Fractal Dimension

Analysis of hyphal branching and fractal dimension was carried out using the program "Fungus", written by Sylvain Delas in conjunction with BioSS. The technical information in the following methodology was obtained from a report of this work (Delas *et al.*, 1996).

3.5.5.1 Program Development

The program was written with the aim of extracting suitable information from the images to help in characterisation of the mycelial geometry, both for whole network measurements such as total hyphal length, and finer detail such as number and length of branches. It also includes the option of calculating additional measurements, such as the number of components and intersections in the network, and global characterisation of the network by the estimation of fractal dimension. As the program was produced as part of a mathematical study into techniques for computerised image analysis of networks composed basically of a series of lines, not all computed parameters are applicable in biological characterisation of the fungal mycelium. The program was designed to run using imported images in bitmap (.BMP) format. Mycelial maps (Section 3.3.4.2) captured as .BMP images via the Q600 image analysis system (Leica, UK) provided the external source of information on which the program was based.

Development of the program was centred on the requirement of extracting necessary and complex information from a digitised image of the arbuscular mycorrhizal fungal mycelium, while maintaining a simple user-interface which did not require specialist knowledge of computers. The program was written in Visual Basic for compatibility with PC based systems running under the Windows 3.1 or Windows 95 environments. The program is presented to the user as a multiple window environment with pull down menus enabling user-choice (Figures 3.5 a-d).

The main algorithms are:

thresholding and skeletonisation;

determination of connected components;

branching calculations, including branch length measurement; measurement of the surface formed by points at a range of distances from the skeleton for evaluation of fractal dimension.

The program encodes the images in a two dimensional array which represents the number of rows and columns present in the image. Each element in the array is allocated a number between 0 and 255, indicating the corresponding grey level. The image is then converted from greyscale to binary by fixing a threshold level between grey level values 0 and 255, and allocating a value of 1 to any pixel darker than this, and 0 to other pixels (these values serve to indicate the presence or absence of a pixel in the binary image, and do not correspond with grey level intensity values of 0 and 1). This produces a new array giving a binary representation of the image which forms the basis of all subsequent calculations. The binary images are first thinned to a single pixel width.

Morphological operations (eg. Serra, 1982; Glasbey & Horgan, 1995) are used in transformation of objects. These operations study the shapes of objects by assuming that an image consists of structures that can be handled by set theory. Object transformation by morphological operations uses small matrices, simple sets or patterns of pixels, known as structuring elements which are designed to achieve a particular operation. In practice, the centre of the structuring element is superimposed on to each pixel in the image and the background pixels covered by the element are investigated. If each pixel corresponds to the background value, the image is left unaltered. Otherwise, the image is amended appropriately with respect to the chosen structuring element. Thus, morphological operations involve exploration of the image by determining where the structuring element will "fit in" to the image, and can be used in transforming an image or measuring its properties by comparing the transformed image with the original. Each of the images can then be viewed as a group of points with a value of 1 on a two dimensional grid which codes the image and provides the basis of subsequent erosion or dilation operations (Gonzalez & Woods, 1992).

Structuring elements can be varied according to the imported image, although the choice of structuring elements is reduced by the necessity of maintaining the number of connecting components. It is thus necessary to impose further constraints on the operations in order to prevent deletion of lines of single pixel thickness, and to prevent reduction of the image to a group of isolated points. If possible, it is also preferable for the operation to be independent of rotation and symmetry, necessitating the use of structuring elements which are indifferent to these transformations. In this case, a structuring element based on a matrix of 3x3 was used, in which each pixel is

allocated a value of 0 (background), 1 (object) or i (indeterminate). See, for example, Figure 3.3 which illustrates eight structuring elements.

The operation central to calculation of the skeleton is termed "Hit or Miss", and is based on a formula relating the total points in the image mask which belong to the object (1) and those belonging to the background (0). In the thinning operation, points from the image that correspond to the structuring element are retained. Any points which are indeterminate are removed. Each point in the image with a value of 1 communicates with points of equivalent value amongst its eight nearest neighbours, giving rise to the term "connected components". The use of the structuring element reduces the number of points of value 1 without altering the number of connecting components in the image in a process termed "thinning out". Calculation of the skeleton is based on this theory, and the image is thinned out by successive applications of the structuring element until no further modifications are obtained.

In this case, the algorithm which enables skeletonisation by reduction of the image structure to a single pixel thickness is based on the following eight structuring elements, in which a pixel and each of its eight neighbours are denoted as 1 (object), 0 (background) or i (indeterminate) (Figure 3.3). The problem is presented in terms of constraints on image simplification ie. it is forbidden to remove a point which is essential for network connections, or which is at the extremity of a hypha. The sum of the structuring elements is then defined by the sum of the constraints.

1	1	1	i	1	i	0	i	1	0	0	i
i	1	i	0	1	1	0	1	1	0	1	1
0	0	0	0	0	i	0	i	1	i	1	i
0	0	0	i	0	0	1	i	0	i	1	i
i	1	i	1	1	0	1	1	0	1	1	0
1	1	1	i	1	i	1	i	0	i	0	0

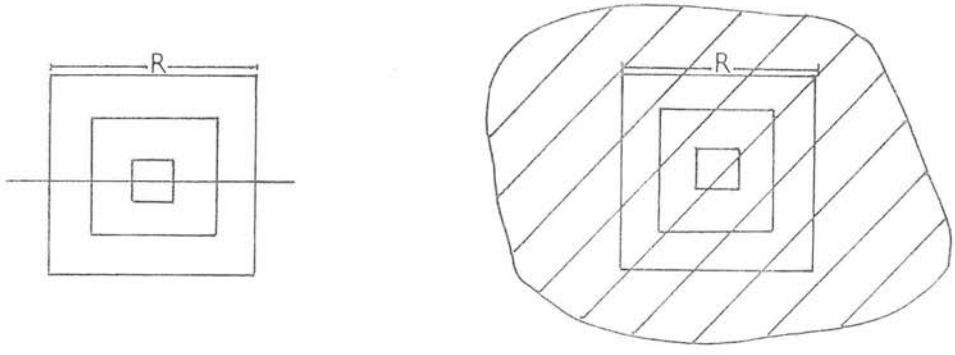
1 = object; 0 = background; i = indeterminate

Figure 3.3: The eight structuring elements forming the basis of the skeletonising algorithm used in the Fungus program

Because mycelial measurements based solely on length do not give any indication of the spatial distribution, the program incorporates a measure of the

surface formed by points at a given distance (δ) from the skeleton, allowing homogeneity of the network to be established and used in calculation of fractal dimension (F.D.). Calculation of the surface using different values of δ necessitates the creation of a table relating any point in the image to its distance from the next nearest point in the network. For this application, calculation of the distance table was based on work by Borgefors (1986).

The fractal concept has been described in Section 3.1.2, and for this purpose can be simply re-iterated as the rate at which the amount of the object visible increases with the size of the viewing window. For a line or a simple curve it increases linearly, while for a solid area it increases as the square of the "box" size (Figure 3.4).



The amount of line in a box of side R proportional to R ie. $A=R^1$

The amount of area in a box of side is R is proportional to R^2 ie. $A=R^2$

Figure 3.4: Graphical definition of the fractal dimension of an object

Because real objects don't have infinitely fine detail, it is necessary to specify the range of scales over which the F.D. will be calculated. This is given as the "box" size (as used in the above definition). The program developed for this study allows both the radii over which the calculations are to be carried out to be specified each time, and the method of calculation to be selected. The two methods used for the calculation of the F.D. are the "box counting" and the "surface in box" methods. The box counting method looks at the number of boxes which include part of the object when the whole image is divided into boxes. When the box size is altered, the number of boxes (N) which touch the object should also change. This can be used to obtain "d" for the equation $N=R^d$. The surface in box method uses the definition directly, and thus counts the areas in each box and finds the power "d" in $A= R^d$ by regression. The fractal dimension is the power of R in relation to $A=R^d$.

As previously discussed, each pixel in the digitised image communicates with its eight nearest neighbours within the network, thus enabling the connecting components of the network to be defined. The Fungus program incorporates an algorithm to calculate the number of these components, and to attribute a label to each pixel representing the number of the component to which it belongs. This algorithm is applied in different places in the program, allowing designation of labels to component parts of the mycelial image and calculations of the size of each component in pixels. This enables the deletion of components smaller than a given size, allowing elimination of background noise from images which are not pre-edited. The numbering of the connected components forms the basis of the numbering of the branches and intersections within the network, and of calculation of branch length.

In writing the program, calculated values were checked using simple examples such as circles and straight networks, and showed an error of a few percent. The greater the number of pixels within any given component, the more precise the results. Programming details are included in Appendix 2.

3.5.5.2 Use of the Program

Traced images of the AM fungal mycelia were converted to .BMP files via the Q600 image analysis system (Leica, UK). The Fungus program was calibrated for each image to obtain the scale in mm/pixel. This was achieved by imaging a 1mm scale bar at the same microscope magnification as that at which the fungal mycelium was originally viewed. The imaged scale bar was calibrated via the Q600, and the calibration value fed into the Fungus program. The image files were stored in a c:drive directory from which they could be read directly into the program.

The program is operated via a series of windows and pull down menu options (Figure 3.5a-d). The opening window has two pull down menu options, **File** and **Run**. Selecting File accesses the File menus:

File - load picture. This menu is used for selection of image files, and can be called several times to create a list of files to be processed in sequence. In practice, files were analysed individually as editing was frequently necessary, and this was more easily carried out on each file immediately after analysis. In addition, each file was individually calibrated, and so the program options had to be reset each time.

File - see list allows the user to view the list of selected files, and allows modification of the list by removal of files if necessary.

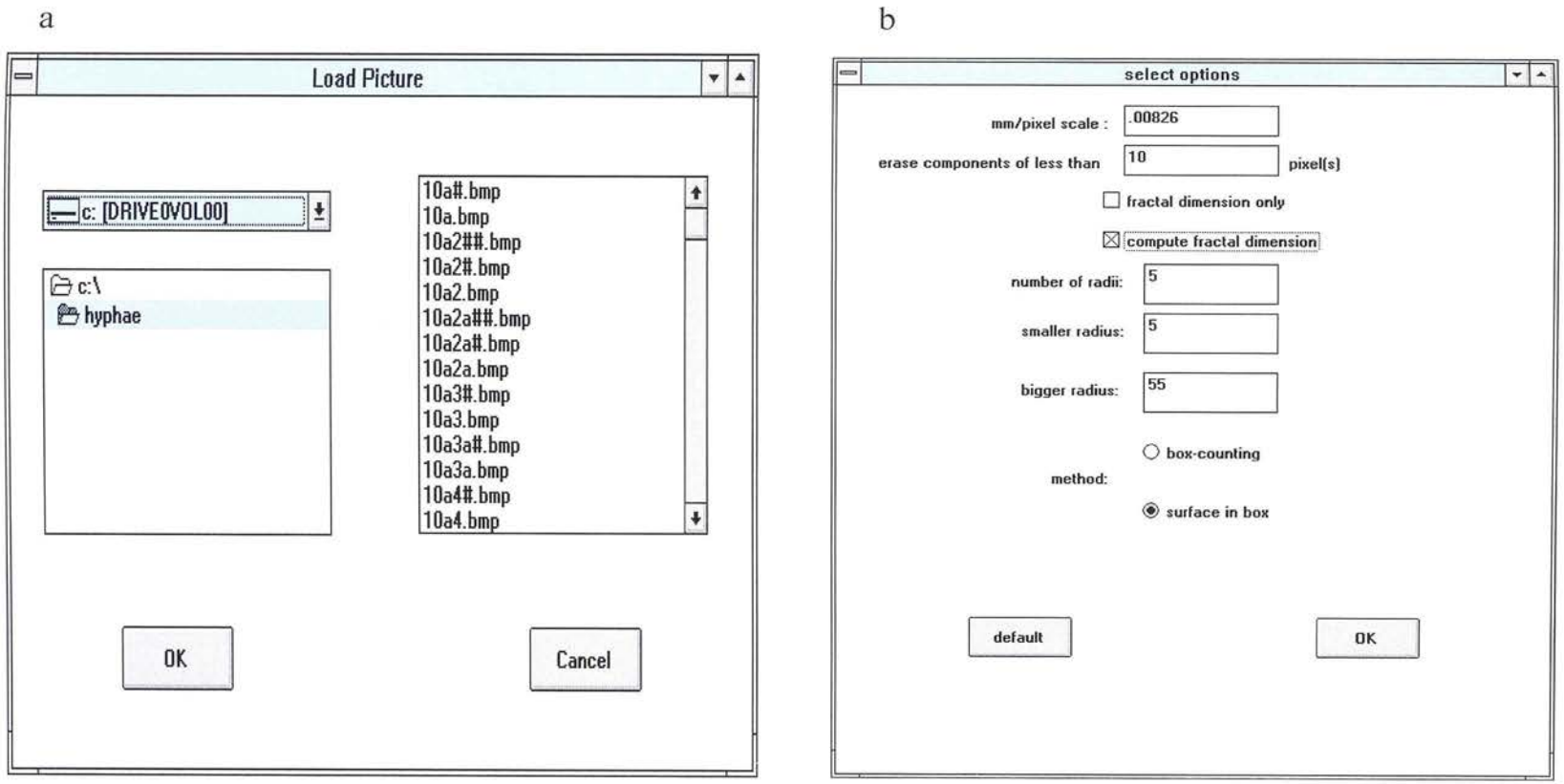


Figure 3.5: (a) File window of the "Fungus" program showing the Load Picture option and the file list
(b) Run window showing program options

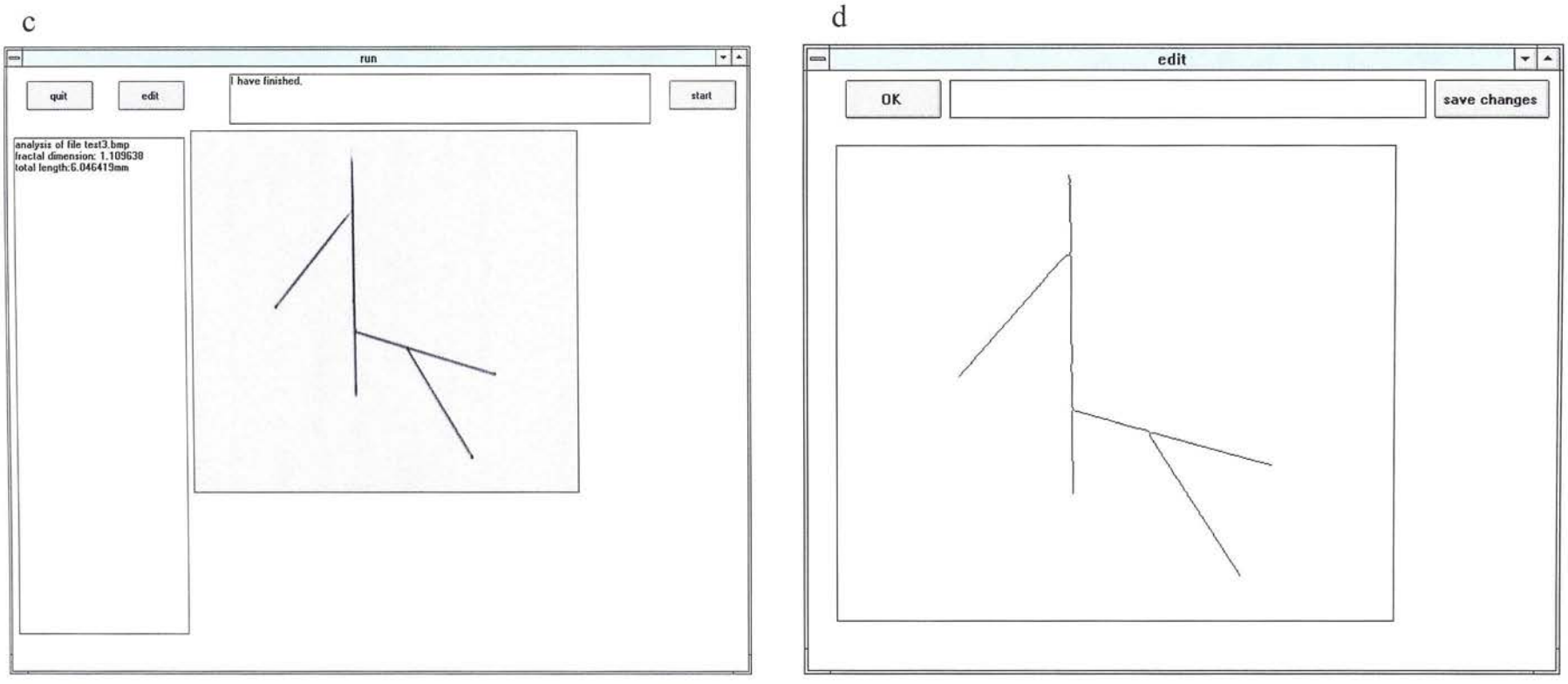


Figure 3.5: (c) Run window of the "Fungus" Program, showing example image and results
(d) Edit window, showing edited version of the image

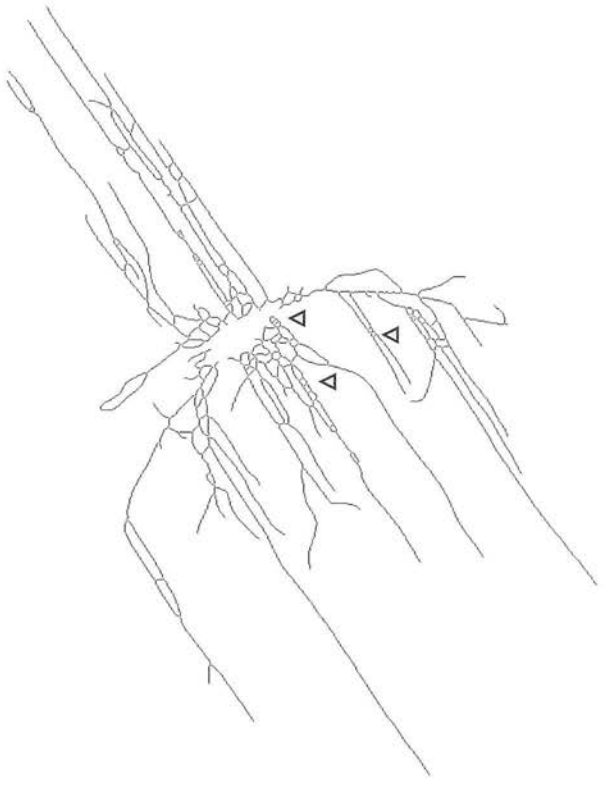
File - exit exits the program

Selecting Run accesses the Run menus:

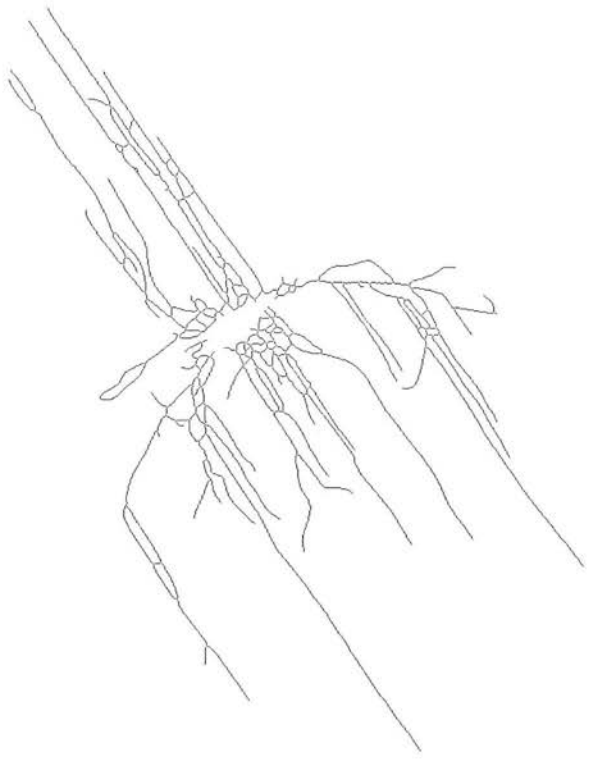
Run - options selects the options window, which gives the choices for running the program. This menu allows the user to calibrate the program for the particular image to be analysed, and to chose between measurement of F.D. only or F.D. plus hyphal measurements. In addition, the radii and choice of calculation method for F.D. are selected here. In this study, the mm/pixel scale was set according to calibration via the Q600 image analysis system (Leica, UK) as described earlier, and five radii were set with a minimum of 5 and a maximum of 55. The "surface in box" method of calculating F.D. was selected because although it is slower than the "box counting" method, it shows a higher degree of accuracy.

Run - go opens the Run window and allows the user to start the calculations or quit and return to the options menu. While the program is running, a small window tells the user which calculation is currently in progress, and a second window accumulates the results. The results are also saved in a text file (Results.txt). In addition, a results image is created and saved. This can be viewed in the skeletonised form via an edit option, which becomes available when the program has finished analysing the image. The edit option also facilitates user intervention, allowing the user to zoom in on the image for pixel by pixel editing using the mouse, and saves the new image. The program can then be re-run on the edited version of the image to give more accurate results. In practice, use of the edit facility was of particular importance due to the complex nature of many of the images (Figure 3.6a & b). In edit mode, moving the mouse over the image highlights and identifies individual components of the image, and shows their length.

Results generated by the program that were used in this study were the number of branches and the F.D. Results for the number of branches required amendment to allow for the number of connected components in the image. The formula used in branch calculations was based on an image in which everything was part of one connected whole. However, in this case, many of the test images were made up of unconnected parts of a mycelium. The 'real' number of branches calculated by the program was amended by a correction factor (actual number of connected components - expected number of components (ie. 1)) to give the actual number of branches. From information provided by the program, the number of branches per mm of hyphal length was calculated.



a



b

Figure 3.6: Skeletonised hyphal images (a) pre- and (b) post-editing
(arrows indicate example areas in which skeleton required editing)

3.6 Results: Development of Experimental Methodologies

The methods used to test moisture retention *in vitro* (Section 3.4; Table 3.3) using dialysis membrane as the substrate for regrowth of mycorrhizal hyphae from root piece inoculum were all able to prevent desiccation of the membranes. Despite evidence of initial rapid growth of hyphae, after 42 day incubation under the test conditions overall hyphal growth was minimal. Contamination problems were also apparent (Table 3.3). Control root pieces inoculated directly onto the dialysis membrane with no overlay showed improved hyphal regrowth over those incubated with an overlay. However, development was slow and colonisation of test plantlets by regrowth hyphae did not occur. Destructive staining of control root pieces incubated between cellulose nitrate filters using the "sandwich" technique showed little hyphal growth after 7 days incubation, but extensive growth after 14 days (Table 3.3). This indicated the potential for *in vitro* regrowth of hyphae from root pieces colonised by AMF under favourable conditions.

Tests using a dialysis membrane "sandwich" (Section 3.4) showed regrowth of hyphae from root pieces within 3 days of inoculation onto the membrane. However, hyphal development was not extensive. Even after removal of the upper membrane to improve visualisation, growth of the hyphae did not exceed 50% of the low power field of view of the binocular microscope (approximately 7.5 x 6 mm) after 35 days. Contamination was also evident on the membranes.

The final tests used root pieces inoculated onto either dialysis membrane between two cellulose nitrate filters, or onto membrane with no overlay moistened with cotton wool pads (Section 3.4). Results indicated that the use of a single uncovered membrane maximised hyphal development. Where the membrane was incubated between two cellulose nitrate filters hyphal growth remained sparse, covering a maximum of 25% of the low power field of view of the binocular microscope. Growth was more extensive when the root piece was inoculated onto the membrane and left uncovered; hyphae were able to extend beyond the full low power field of view within a three week period. Experiments were run for a maximum of 3 weeks. Where the mycelium extended beyond the microscope field of view, overlapping images were recorded and the full mycelium reconstructed manually to recreate the full mycelial map.

Table 3.3 Hyphal regrowth from AMF colonised root pieces incubated with different substrates to test moisture retention capabilities

Substrate	Time (d)		
	7	14	42
Capillary matting + dialysis membrane (DM)	Hyphal regrowth from 66% of root pieces	Extensive hyphal growth	Maximum growth equivalent to single microscope field of view at low power
Cellulose nitrate (CN) filter sandwich	Little hyphal regrowth from single root piece	Extensive hyphal regrowth	-
Phytagel + DM	No hyphal regrowth. Contamination on membrane	Little hyphal growth. Contamination on membrane	Contamination. Hyphae no longer evident
Washed gravel + DM	Extensive hyphal regrowth from 66% of root pieces	Extensive hyphal growth	Contamination on membrane
Washed gravel + CN filter + DM	Regrowth hyphae from 33% of root pieces. Extensive growth from single piece	Limited hyphal growth	Hyphal growth quite extensive

3.7 Results: Experimental Procedures

3.7.1 Control Experiments

The first experiment was aimed at testing both for any background effect of agarose as a substrate for carrying test compounds, and for any possible effect of root piece orientation on hyphal growth. This experiment thus examined hyphal regrowth from root piece inoculum both in the absence of agarose and in the presence of agarose minus test compounds. It also tested the effect of uni-directional placement of root piece inoculum relative to membrane striations. A further test was included to examine the effect of host root exudates on hyphal growth and orientation, using two exudate impregnated blocks (Section 3.5.2) placed at diagonals 5mm from the root piece (Section 3.5.1).

No significant difference was found between hyphal growth from root pieces incubated in the absence of agarose and those incubated with agarose minus a test compound. This indicated that agarose was a satisfactory carrier for test compounds, and had no independent effect on hyphal growth (Table 3.4). Specific orientation of root pieces did not significantly affect hyphal growth (Table 3.4), despite apparently greater values for hyphal length. Statistical analysis was carried out using the Kruskal-Wallis test for group comparisons, and the Mann-Whitney test for comparison of pairs. These are non-parametric tests suited to data which does not fall within a normal distribution, and are therefore appropriate for variable data sets. The tests were carried out using the Minitab for Windows statistics package (version 10; Minitab Inc., USA). Following Kruskal-Wallis analysis, significances were tested using simultaneous rank tests. Statistical analysis of these data using the Kruskal-Wallis test indicated that differences between controls were significant at $p<0.05$ (actual value 0.049). However, this value was not sufficiently less than 0.05 to withstand further testing, and application of simultaneous rank tests did not highlight any specific differences.

Table 3.4 Hyphal regrowth (mm) from AMF colonised root pieces in control experiments and in the presence of host plant root exudates

Figures are means of 3 to 4 replicates. Figures in brackets are SE of means

Treatment	Time (d)	Hyphal length (mm)
Control A	7	3.8 (1.75) [a]
Control B	7	15.8 (13.50) [a]
Control C	7	70.4 (21.40) [a]
H Exudate	7	3.1 (0.57) [b]

Figures with the same letter within each data set are not significantly different
Control A = No agarose
Control B = Agarose minus test compound
Control C = Orientated root piece
H Exudate = Host plant root exudate

In subsequent experiments root pieces were always placed uni-directionally on the membrane, in order to maintain uniformity of experimental conditions. In addition, to try and counteract variability problems, separate controls were run with each set of experiments. This ensured that all root pieces used within a particular set of experiments originated from the same root sample.

Hyphal growth in the presence of host root exudates was significantly lower ($p<0.05$) than in the control experiment using specifically oriented root pieces (Mann-Whitney test, $p=0.03$; Table 3.4). No directional responses, which may have indicated chemotaxis, were apparent.

3.7.2 Effects of Natural and Synthetic Plant-Derived Compounds

3.7.2.1 Comparisons of Root Exudates and Plant Flavonoids

In the first of this series of experiments the treatments were imposed seven days after inoculation of the root piece onto the membrane. Measurements of hyphal growth were taken on day seven, prior to addition of agarose blocks impregnated with either host or non-host plant root exudates, and on day 14, seven days after the treatments were imposed.

As expected, no significant differences were found between treatments prior to addition of the test compounds (Table 3.5) as at this stage all treatments were essentially controls. The second measurements were taken after 14 days, when test compounds had been in place for seven days. No significant differences in hyphal growth were found between the control, host exudates and non-host exudates, or between the two treatments themselves (Table 3.5). SEM's were again high, therefore it is also possible that treatment effects are masked by within treatment variability.

Table 3.5 Hyphal regrowth (mm) from AMF colonised root pieces in the presence of host plant and non-host plant root exudates

Figures are means of 5 replicates. Figures in brackets are SE of means

Treatment	Time (d)	Hyphal length (mm)
Control	7	53.3 (33.5) [a]
H Exudate	7	91.6 (41.9) [a]
NH Exudate	7	121.1 (67.2) [a]
Control	14	105.2 (67.1) [a]
H Exudate	14	223.1 (75.0) [a]
NH Exudate	14	163.3 (85.7) [a]

Figures with the same letter within each data set are not significantly different
H Exudate = Host plant root exudate
NH Exudate = Non-host plant root exudate

Following these observations, treatments were imposed at the start of subsequent experiments. In addition, since chemotactic responses were not evident, compounds were tested in a homogeneous environment using Petri dishes containing 20 ml agarose impregnated with the test compound. Mycelial images were recorded after seven and 14 days.

Independent comparisons of each treatment with the control (Mann-Whitney test) indicated that after seven days both non-host exudates and the flavonoid naringenin were having significantly depressive effects on mycelial growth (Table 3.6: non-host exudate $p=0.050$; naringenin $p=0.014$). In contrast, neither host root exudates nor hesperetin had significant effects on hyphal growth (Table 3.6).

Kruskal-Wallis analysis of hyphal lengths at $t=14$ resulted in a p value of 0.054, which did not withstand further testing for significant differences between treatments. Re-analysis of pairs using the Mann-Whitney test indicated that naringenin significantly depressed hyphal growth relative to the control ($p=0.016$).

Table 3.6 Hyphal regrowth (mm) from AMF colonised root pieces in the presence of host and non-host plant root exudates and plant flavonoids

Figures are means of 5 to 10 replicates. Figures in brackets are SE of means

Treatment	Time (d)	Hyphal length (mm)
Control	7	6.1 (2.02)[a]
H Exudate	7	4.5 (2.95)[a]
NH Exudate	7	1.9 (1.04)[b]
Hesperetin	7	3.8 (1.99)[a]
Naringenin	7	1.2 (0.60)[b]
Control	14	10.1 (3.70)[a]
H Exudate	14	45.4 (27.70)[a]
NH Exudate	14	5.4 (3.09)[a]
Hesperetin	14	2.8 (1.33)[a]
Naringenin	14	1.6 (0.67)[b]

Figures with the same letter within each data set are not significantly different
H Exudate = Host plant root exudate
NH Exudate = Non-host plant root exudate

Although attempts were made to produce exudates from colonised host plants for use in these experiments (Section 3.5.2.1), contamination problems were

encountered each time the system was set up. Thus, this section does not contain information concerning the exudates of colonised host plants, and it is recognised that this would be an important area for future work.

A 50:50 mixture of host:non-host exudate was also tested, and was found to have no significant influence on hyphal growth.

3.7.2.2 Effects of Root Extracts on Hyphal Growth

The effects of root extracts were similarly tested, using agarose impregnated with extract as a homogeneous growth medium. Root extracts of both colonised and non-colonised host plants (cucumber) and non-host plants (oilseed rape) were tested for their influence on hyphal growth over a period of up to 14 days.

Table 3.7 Hyphal regrowth (mm) from AMF colonised root pieces in the presence of colonised and non-colonised host and non-host root extracts

Figures are means of 2 to 11 replicates

Treatment	Time (d)	Hyphal length (mm)
Control	7	19.4[a]
N-C H Extract	7	43.7[a]
Control	7	436.3[a]
NH Extract	7	6854.1[a]
✧ Control	7	235.8[a]
CH Extract	7	1179.8[b]
Control	14	32.9[a]
N-C H Extract	14	73.1[a]

Figures with the same letter within each data set are not significantly different
✧ Test species *Gigaspora rosea*
N-C H Extract = Non-colonised host plant root extract
NH Extract = Non-host plant root extract
CH Extract = Colonised host plant root extract

Neither uncolonised host or non-host root extracts significantly influenced mycorrhizal hyphal growth. Uncolonised cucumber root extracts had no significant effect on hyphal growth after 14 days under test conditions (Mann-Whitney test t=7, p=0.065; t=14, p=0.0787; Table 3.7). Similarly non-host root extracts, tested for only a seven day period due to the extent of hyphal growth, did not significantly affect growth relative to the control (Mann-Whitney test p=0.081; Table 3.7). In contrast,

colonised cucumber root extracts significantly increased hyphal growth after seven days (Mann-Whitney test p=0.037; Table 3.7).

3.7.2.3 Heat Stability of Exudates

As an initial step in attempting to identify potentially active constituents, heat stability tests were carried out. Cucumber and oilseed rape exudates were heat treated by autoclaving at 121°C and 15 psi for 20 minutes prior to adding them to agarose as the substrate. Controls were also treated in this way. Heat treatment did not significantly affect hyphal growth for any of these test substances (Krukall-Wallis test p=0.69; Table 3.8), indicating that any active compound present was not influenced by heat treatment.

Table 3.8 Hyphal regrowth (mm) from AMF colonised root pieces in the presence of host and non-host root exudates ± heat treatment

Figures are means of 5 replicates

Treatment	Time (d)	Hyphal Length (mm)
Control	7	20.2[a]
HT Control	7	10.0[a]
H Exudate	7	42.9[a]
HT Exudate	7	90.3[a]
NH Exudate	7	76.1[a]
HT Exudate	7	23.9[a]

Figures with the same letter within each data set are not significantly different
HT Control = Heat-treated control
H Exudate = Host plant root exudate
NH Exudate = Non-host plant root exudate
HT Exudate = Heat-treated plant root exudate

3.7.3 Exudate Fractionation and AM Fungal Bioassays

The effects on hyphal growth of <3K, 3-10K and >10K molecular fractions of six test substances were assessed visually on gridded cellulose nitrate filters as described in Section 3.5.3. Due to unequal sample sizes (ie. unbalanced data sets) it was not possible to analyse this data using two-way analysis of variance (ANOVA). This method of analysis would otherwise have been used to highlight any differences within test substances and fractions of these, and any interactions between the two.

In order to look at differences in effect on fungal growth both between fractions of a particular test substance, and between test substances for a given

fraction, two one-way ANOVA's were carried out on the data (Minitab for Windows Version 10; Minitab Inc., USA). Differences were calculated by comparison of means using Least Significant Difference (LSD).

Analysis of the effects of molecular fraction for each test substance showed that the >10K fraction did not significantly affect hyphal growth for any of the test substances (Table 3.9). In contrast, both the <3K and 3-10K fractions were able to significantly influence hyphal growth (Table 3.9). Analysis of bioactivity data showed that growth in the presence of the <3K fraction of both the host and colonised host root extracts was significantly less than in the control ($p<0.01$; Table 3.9), suggesting the possibility of fungal growth inhibition. Growth with this fraction of the host extract was also less than with either the host or non-host exudate ($p<0.01$; Table 3.9). Growth effects were more pronounced with the host than the non-host extract. Thus, growth with the <3K fraction of the host extract was significantly less than with the non-host extract ($p<0.01$; Table 3.9). It appears that the <3K fraction may contain inhibitory compounds which do not necessarily show evident effects when the whole sample is applied. Host and non-host exudates had no significant effect, nor were there any significant differences between host and non-host exudates or between host and colonised host root extracts (Table 3.9).

Analysis of the bioactivity of the 3-10K fraction similarly showed significant effects. This fraction of the non-host exudate significantly suppressed hyphal growth relative to the control ($p<0.05$; Table 3.9). In contrast, the non-host extract significantly increased growth relative to the control ($p<0.05$; Table 3.9). The host exudate, extract and colonised extract had no significant effects (Table 3.9). There was no significant difference in growth between host and non-host exudates or extracts, or between host extracts and colonised host extracts. Growth in the presence of this fraction of both the host and colonised host extracts was significantly greater than with either the host or non-host exudates ($p<0.01$; Table 3.9). Extracts were in general more stimulatory than exudates.

It appears from these results that the 3-10K fraction is the most active, and the >10K fraction the least active.

Analysis of the effects of each of the three molecular fractions within a test compound showed significant effects occurring between fractions in the control, host exudate, non-host exudate and colonised host root extract. There were no significant effects of fraction for either the host or non-host extract (Table 3.9).

The control showed significant differences in effect between the <3K fraction and both the 3-10K ($p<0.01$; Table 3.9) and >10K ($p<0.05$; Table 3.9) fractions, but no differences between the 3-10K and >10K fractions.

With the host exudate, growth with the <3K fraction was significantly greater than in the 3-10K fraction ($p<0.01$; Table 3.9). Growth with the >10K fraction was also significantly greater than with the 3-10K fraction ($p<0.05$; Table 3.9). There was no significant difference in growth between the <3K and >10K fractions. This suggests the possibility of a suppressive effect of the 3-10K fraction. Similarly, with the non-host exudate growth was least with the 3-10K fraction, and was significantly less than with both the <3 and >10K fractions ($p<0.01$; Table 3.9). There was no significant difference between the <3K and >10K fractions.

Table 3.9 Hyphal regrowth (mean score) from colonised root pieces in the presence of molecular fractions of root exudates and extracts

Treatment	< 3K Fraction	3-10K Fraction	> 10K Fraction
Control	$4.50^{a☆}$	$2.40^{a◇}$	$3.00^{a◇}$
H Exudate	$3.88^{a☆}$	$0.80^{a◇}$	$3.00^{a☆}$
N-H Exudate	$4.00^{a☆}$	$0.20^{b◇}$	$3.00^{a☆}$
H Extract	$1.75^{b☆}$	$3.80^{a☆}$	$3.20^{a☆}$
N-H Extract	$4.25^{a☆}$	$4.60^{b☆}$	$4.20^{a☆}$
CH Extract	$3.00^{b☆}$	$3.60^{a☆◇}$	$5.20^{a◇}$

Figures with the same letter within each column are not significantly different from the control.
Full descriptions of differences between other treatments are given in the accompanying text
Figures with the same symbol within each row are not significantly different
H Exudate = Host plant root exudate
N-H Exudate = Non-host plant root exudate
H Extract = Host plant root extract
N-H Extract = Non-host plant root extract
CH Extract = Colonised host plant root extract

For the colonised host root extract, growth with the >10K fraction was significantly greater than with the <3K fraction ($p<0.05$). There was no significant difference between the <3K and 3-10K fractions, or between the 3-10K and >10K fractions.

3.7.4 Exudate Analysis

3.7.4.1 Protein and Amino Acid Analysis

Protein absorbance spectrophotometry indicated the presence of proteins in all samples of root extracts and exudates. Amino acid analysis was then carried out using HPLC (Section 3.5.4.1). The standards used for comparison with host and non-host extracts and exudates, and colonised host root extracts, proved to be unsuitable, therefore identification of individual amino acids was not possible and differences were distinguished using peak retention times rather than compound names. The control trace (Figure 3.7a) showed the presence of a number of amino acids, indicating that the sorbarod (Horticultural Sorbarods, Ilacon Ltd., UK) used as the plant growth support contained some amino acids itself in quantities similar to those present in host and non-host exudates. Host exudate profiles showed the presence of seven distinct compounds (Figure 3.7b) all of which also appeared in the control trace (Figure 3.7a). The non-host exudate gave similar results (Figure 3.7c).

Root extracts contained a larger number of compounds and peak heights indicated their presence in greater quantities. The host extract (Figure 3.7d) appeared to contain more amino acids present than the non-host (Figure 3.7e). There seemed to be little difference in amino acid profiles of host and colonised host root extracts (Figures 3.7f & g).

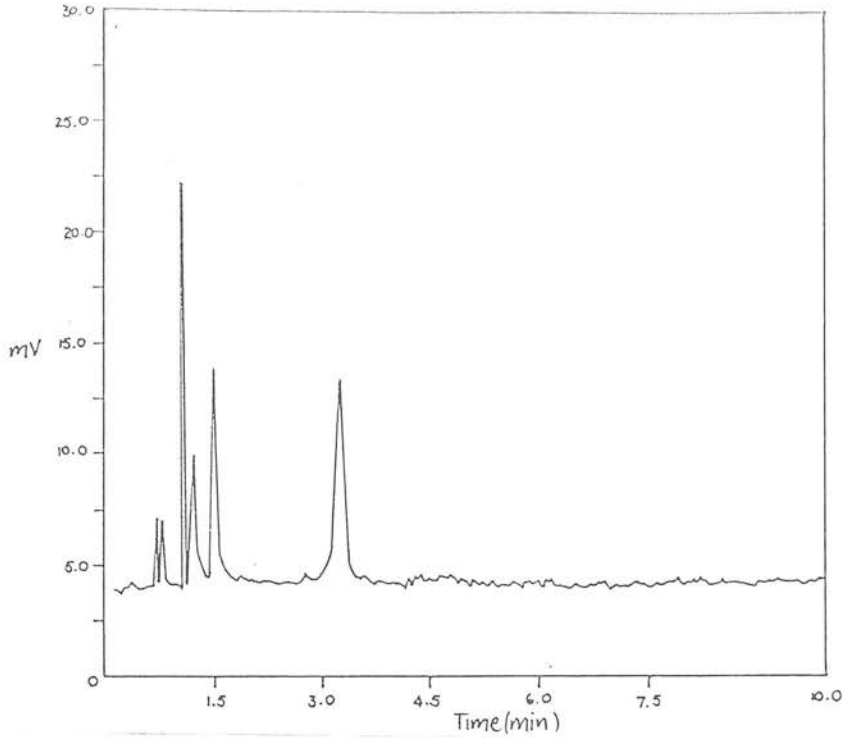
In a second run comparing non-colonised host root extracts and exudates with appropriate amino acid standards, the host root extract again contained a considerably higher number of amino acids than did the exudates (Figure 3.8a & b). All those amino acids present in the exudate were also present in the extract. All were present at a much higher concentration in the extract than in the exudate (Figure 3.8a & b).

Profiles obtained for host extract samples after amino acid hydrolysis were similar to those described above. Host and non-host extract profiles were also very similar (Figure 3.9b & c), the only difference being the presence of alanine in the host, but not in the non-host. Control samples (Figure 3.9a) also contained all the amino acids present in the root samples, with the addition of methionine, although in most cases these were present in lower quantities (Table 3.10). The exceptions were valine, alanine, phenyalanine and lysine, which were present at higher concentrations in the control than in the non-host sample. These results again indicate that the sorbarods (Horticultural Sorbarods, Ilacon Ltd., UK) used for plant growth and extraction of exudates also contained amino acids.

Figure 3.7 a-c: HPLC amino acid profiles of

(a) control
(b) host root exudate
(c) non-host root exudate

(a)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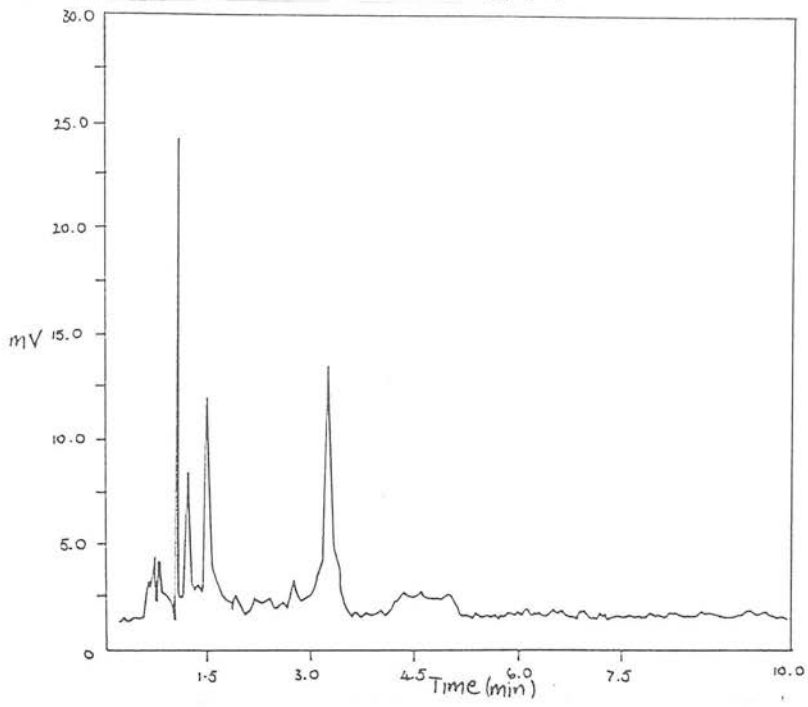
25.0
20.0
mV 15.0
10.0
5.0
0
1.5
3.0
4.5
6.0
7.5
10.0
Time (min)

(b)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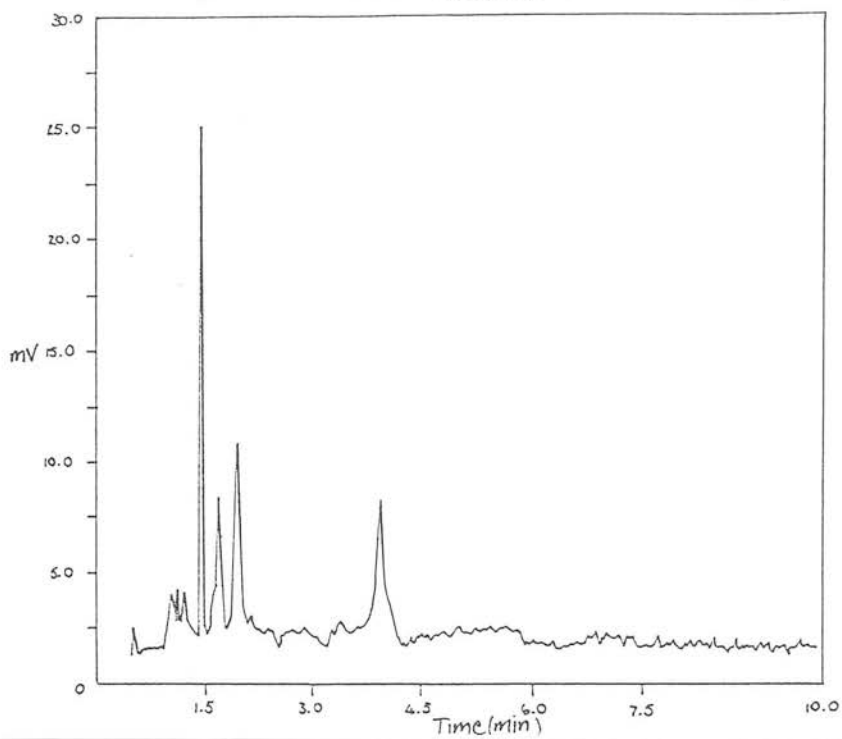
25.0
20.0
mV 15.0
10.0
5.0
0
1.5
3.0
4.5
6.0
7.5
10.0
Time (min)

(c)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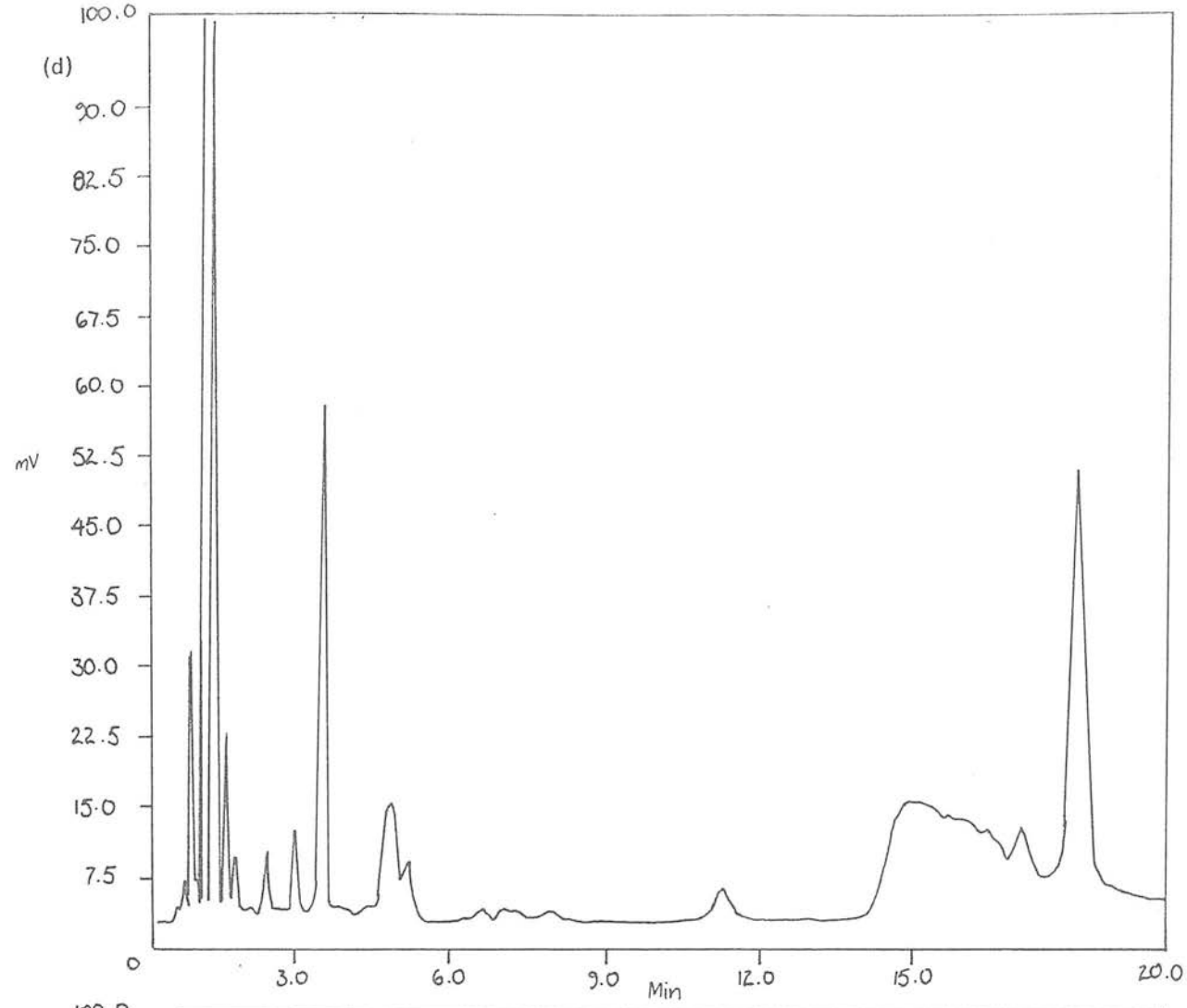
25.0
20.0
mV 15.0
10.0
5.0
0
1.5
3.0
4.5
6.0
7.5
10.0
Time (min)

Figure 3.7 d-e: HPLC amino acid profiles of (d) host root extract
(e) non-host root extract


(d)
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90.0
82.5
75.0
67.5
60.0
mV
52.5
45.0
37.5
30.0
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6.0
9.0
Min
12.0
15.0
20.0

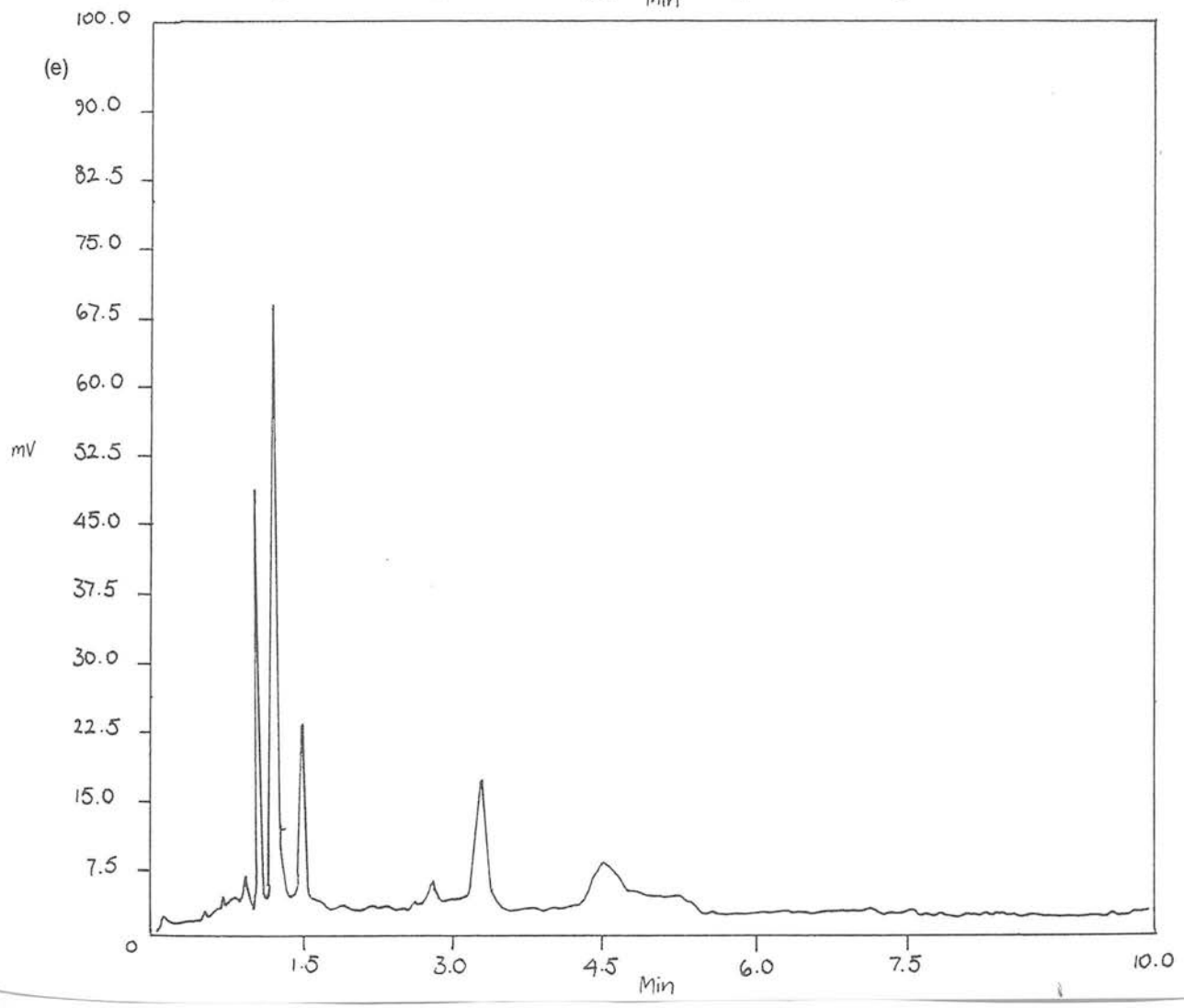
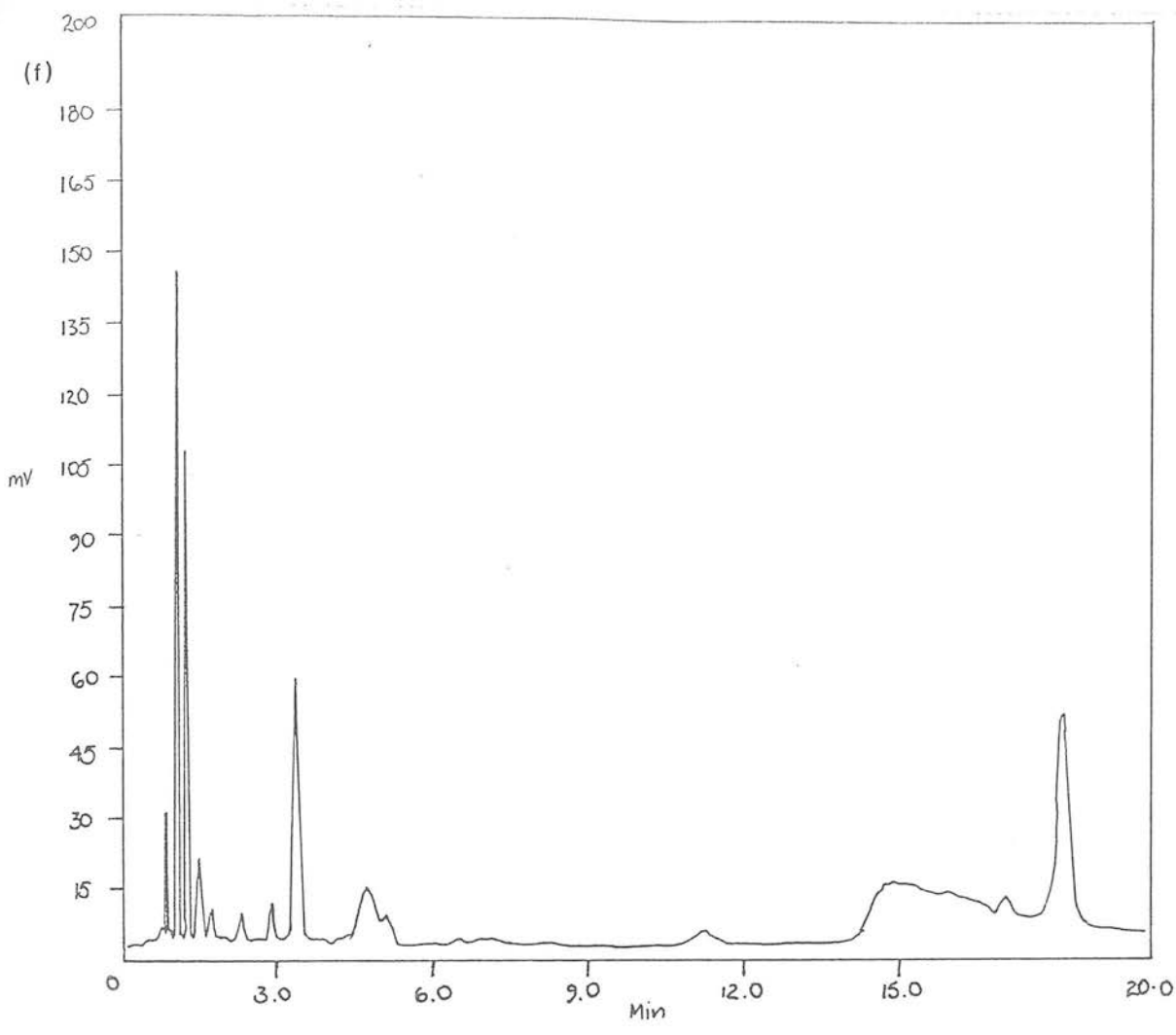

(e)
100.0
90.0
82.5
75.0
67.5
60.0
mV
52.5
45.0
37.5
30.0
22.5
15.0
7.5
0
1.5
3.0
4.5
Min
6.0
7.5
10.0

Figure 3.7 f-g: HPLC amino acid profiles of

(f) host root extract
(g) colonised host root extract


(f)
200
180
165
150
135
120
105
90
75
60
45
30
15
mV
0
3.0
6.0
9.0
12.0
15.0
20.0
Min

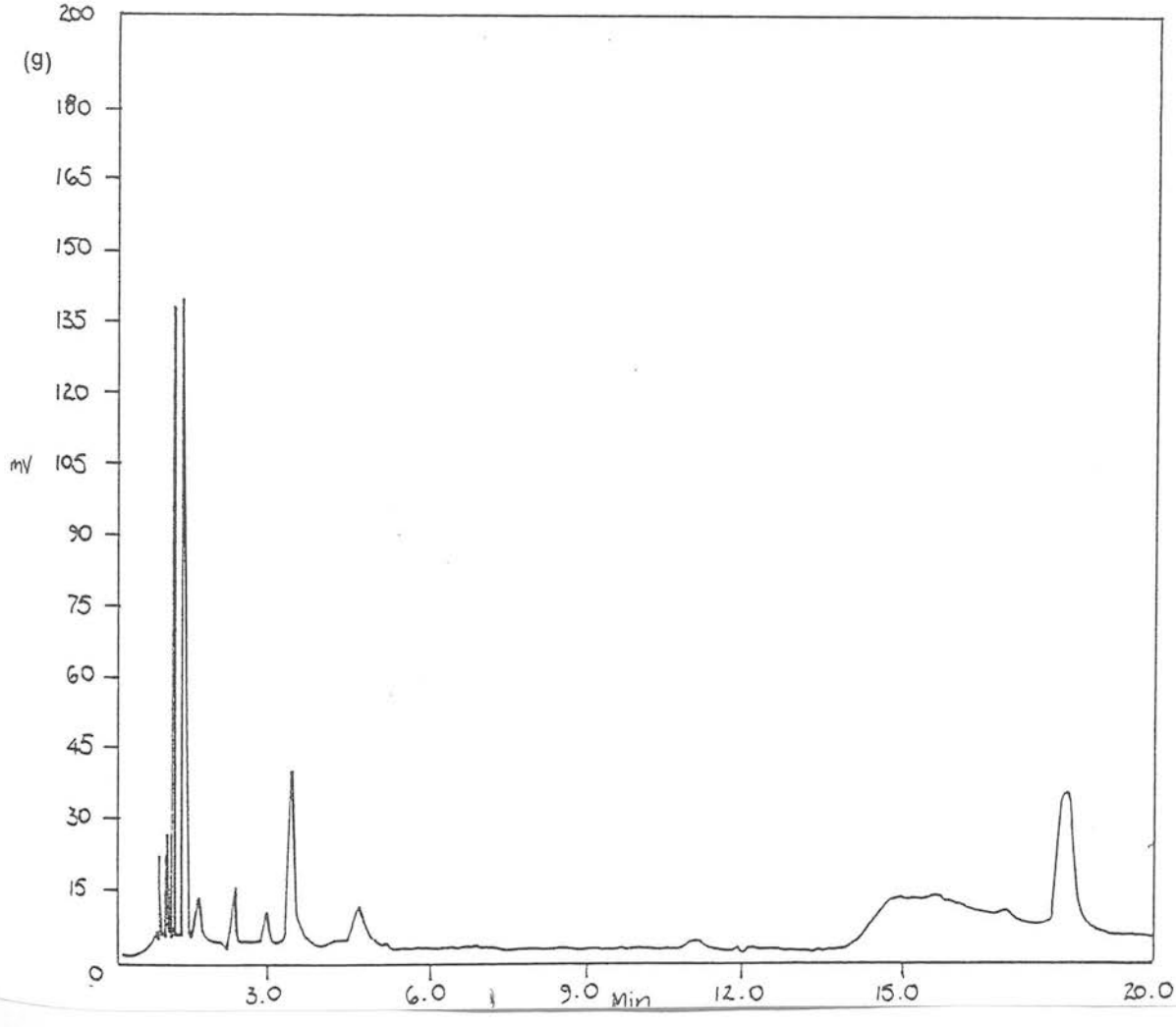
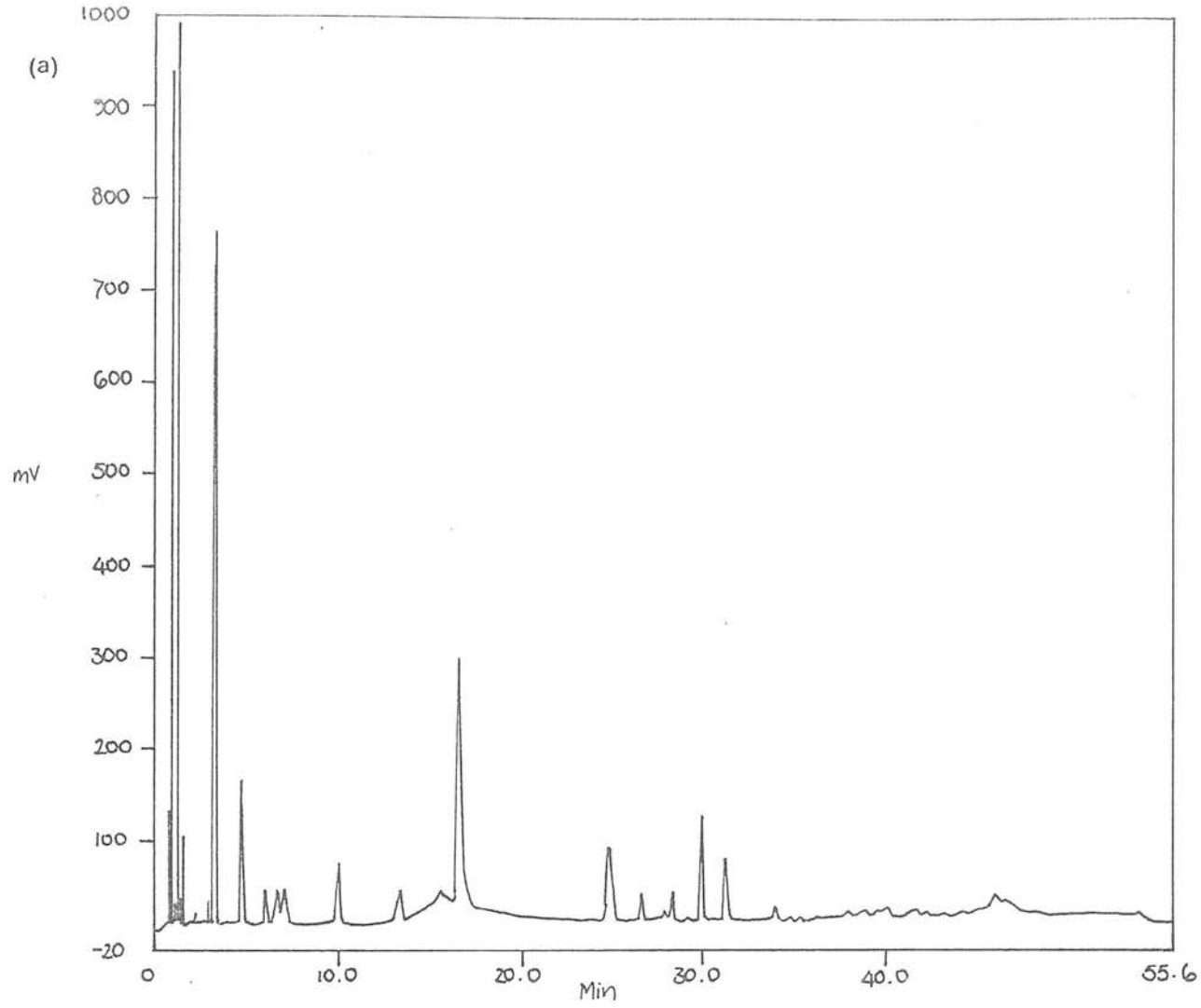

(g)
200
180
165
150
135
120
105
90
75
60
45
30
15
mV
0
3.0
6.0
9.0
12.0
15.0
20.0
Min

Figure 3.8: HPLC amino acid profiles of (a) host root extract
(b) host root exudate


(a)
1000
900
800
700
600
mV
500
400
300
200
100
-20
0
10.0
20.0
30.0
40.0
55.6
Min

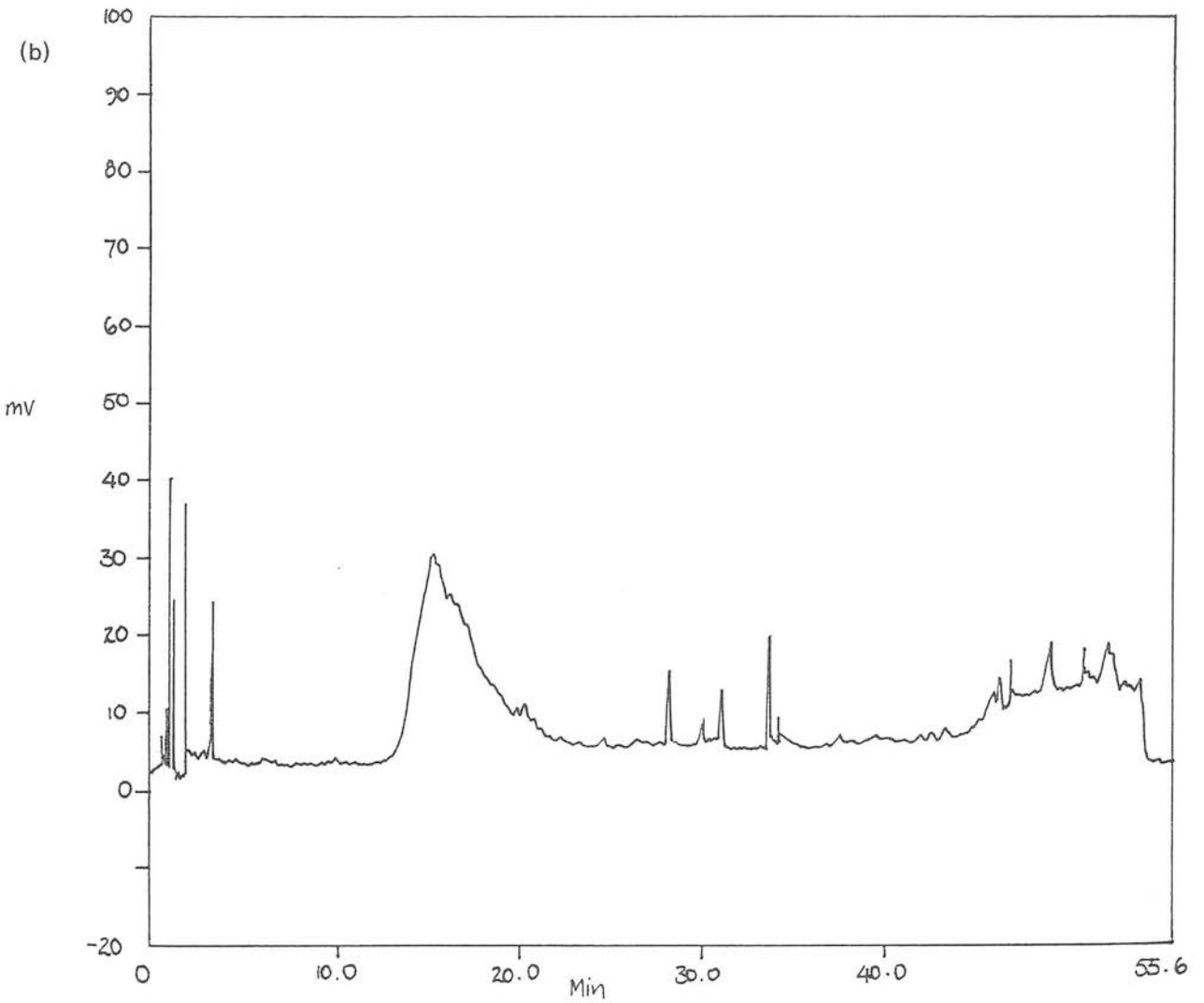

(b)
100
90
80
70
60
mV
50
40
30
20
10
0
-20
0
10.0
20.0
30.0
40.0
55.6
Min

Figure 3.9: HPLC profiles of hydrolysed amino acids of (a) control
(b) host root extract
(c) non-host root extract

Key to amino-acid abbreviations:

As	Aspartic Acid
Gl	Glutamic Acid
Se	Serine
Hi	Histidine
Gly	Glycine
Th	Threonine
Ar	Arginine
Al	Alanine
Ty	Tyrosine
Me	Methionine
Va	Valine
Ph	Phenylalanine
i-Le	iso-Leucine
Le	Leucine
Ly	Lysine

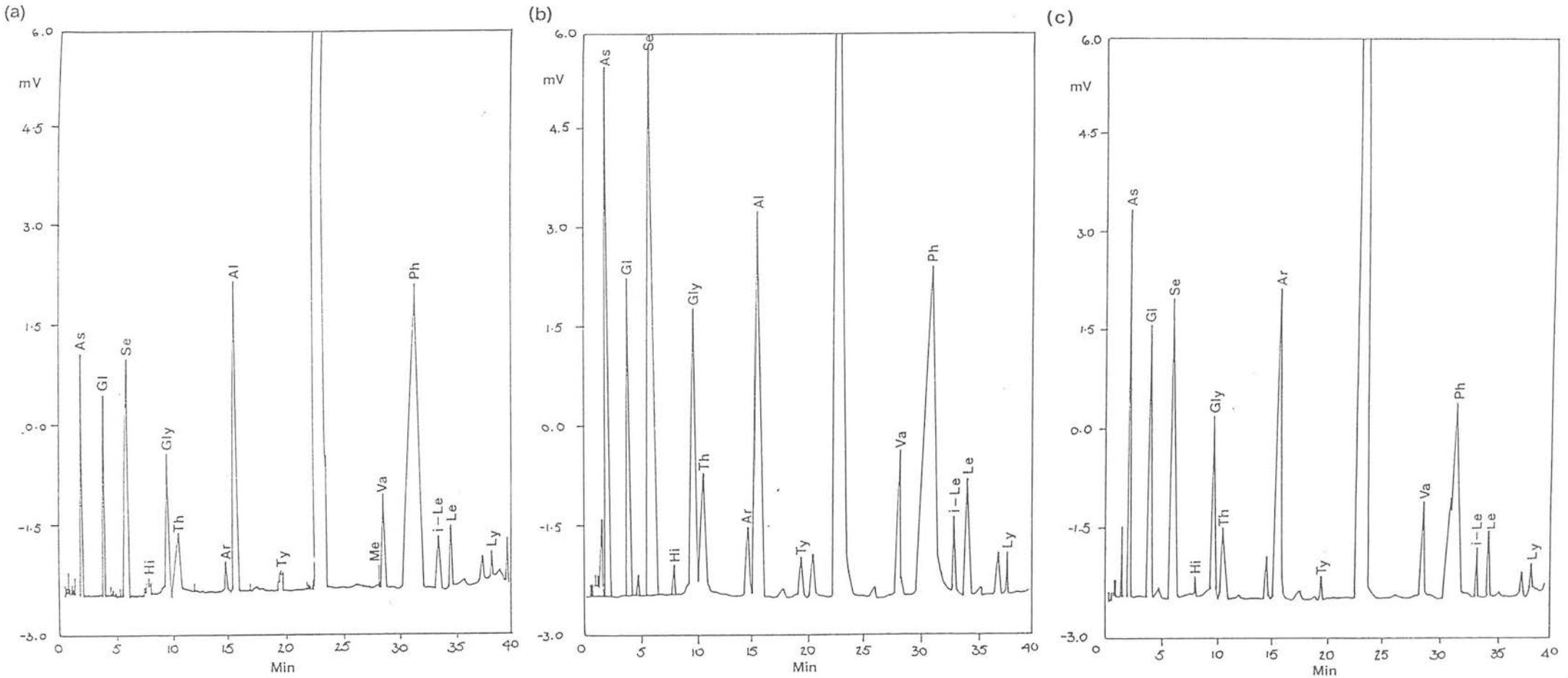

(a)
(b)
(c)
mV
6.0
4.5
3.0
1.5
0.0
-1.5
-3.0
0
5
10
15
20
25
30
35
40
Min
As
Gl
Se
Hi
Gly
Th
Ar
Al
Ty
Me
Va
Ph
i-Le
Le
Ly

Table 3.10 Amino acid profiles (ppb) of host, non-host and control from HPLC of samples after acid hydrolysis

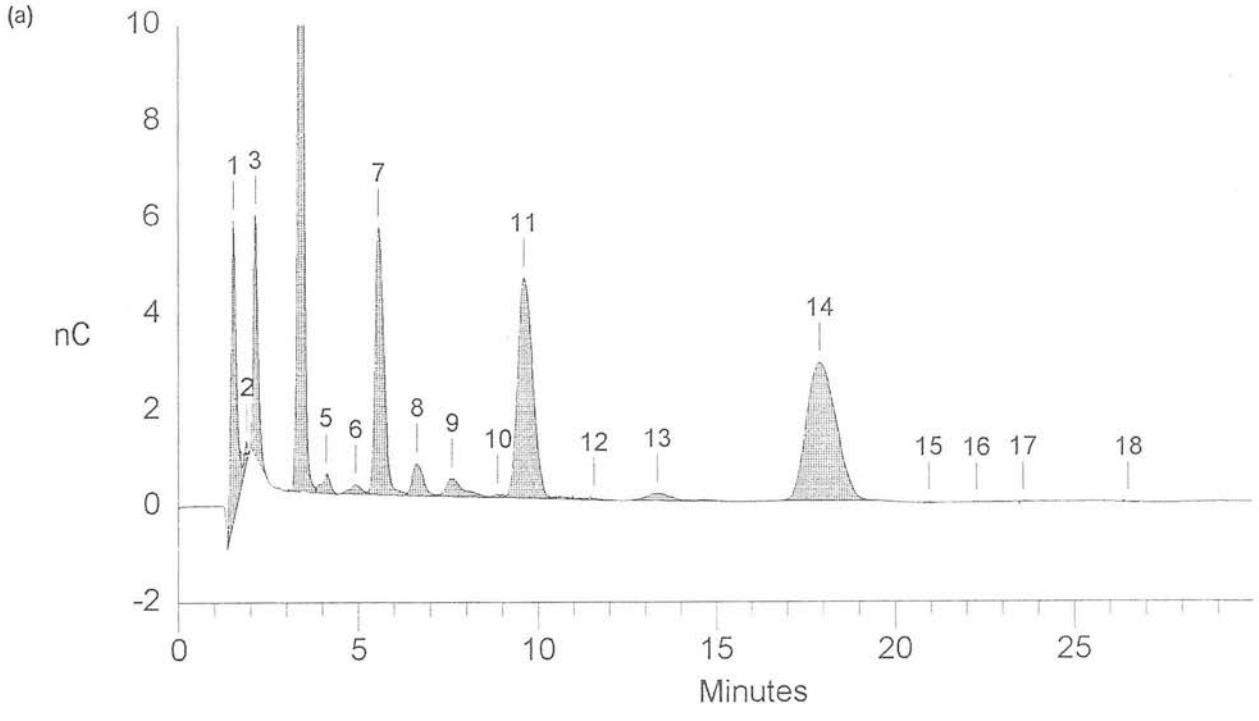
Amino Acid	Quantity (ppb)		
	Control	Host	Non-Host
Aspartic acid	105.30	213.40	150.41
Glutamic acid	112.48	174.62	154.30
Serine	117.40	273.41	151.56
Histidine	16.43	30.27	20.89
Glycine	84.17	165.01	116.90
Threonine	58.17	83.55	79.33
Arginine	32.88	64.96	305.37
Alanine	210.49	250.64	-
Tyrosine	22.01	51.70	35.25
Methionine	5.47	-	-
Valine	65.10	93.56	57.17
Phenylalanine	510.34	596.30	337.94
i-leucine	31.96	64.45	33.80
Leucine	53.55	99.15	53.91
Lysine	68.36	76.79	60.63

3.7.4.2 Carbohydrate Analysis

As with the amino acid analysis, carbohydrate analysis also showed the presence of compounds in the control trace (Figure 3.10a). 18 compounds were recorded over a 30 minute period. Many of these were present in negligible quantities and did not subsequently appear in analysis of test substances. Glucose and sucrose were identified from standards. 18 compounds were also recorded during analysis of the autoclaved control sample. The carbohydrate profile was similar, but differences in peak heights indicated relative differences in quantities of the various compounds present (Figure 3.10b).

Analysis of the cucumber (host) root exudate recorded only two major compounds, of which one was identified as glucose through the use of a glucose standard (Figure 3.10c). Both of the compounds appeared to be present in the control, but in much lower concentrations (ie. lower peak heights on the trace). In contrast, the oilseed rape (non-host) exudate contained a much larger number of compounds. Twenty compounds were eluted, one of which was again identified as

Figure 3.10: Carbohydrate profiles of (a) control
(b) autoclaved control


(a)
10
8
6
4
2
0
-2
nC
1
3
2
7
11
14
5
6
8
9
10
12
13
15
16
17
18
0
5
10
15
20
25
Minutes

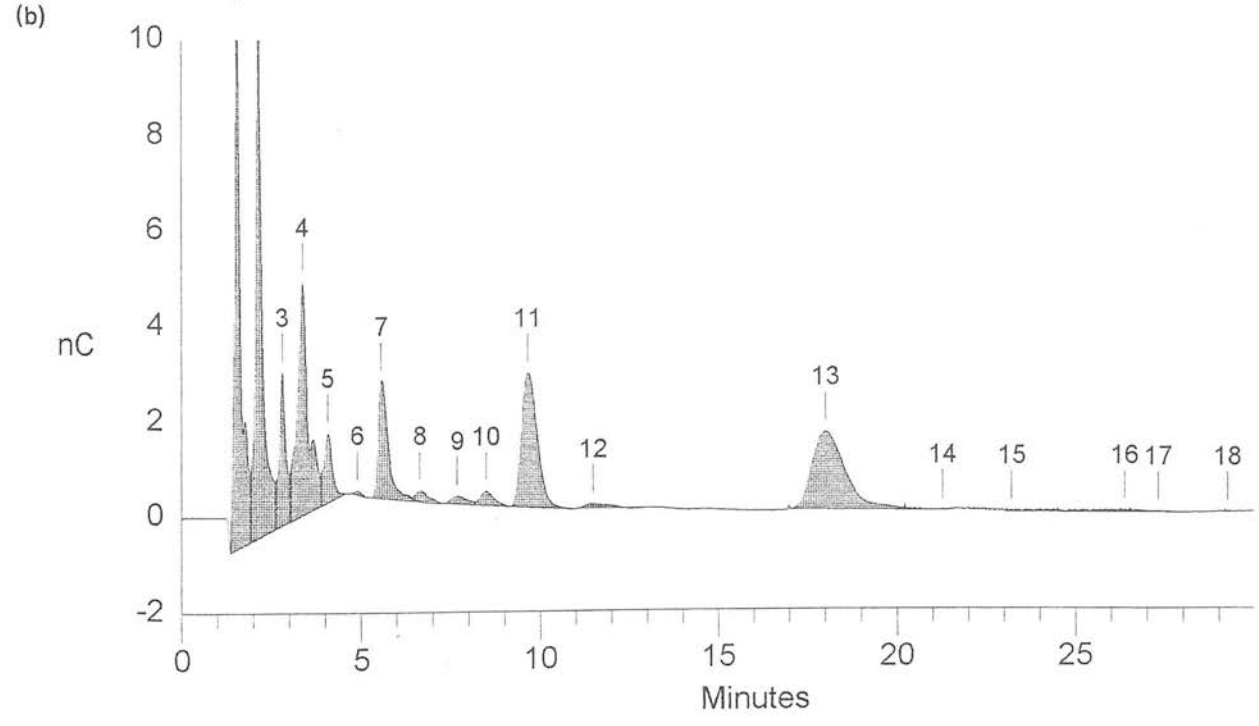

(b)
10
8
6
4
2
0
-2
nC
4
3
7
11
5
13
6
8
9
10
12
14
15
16
17
18
0
5
10
15
20
25
Minutes

Figure 3.10: Carbohydrate profiles of

(c) host root exudate
(d) non-host root exudate
(e) autoclaved host root exudate
(f) autoclaved non-host root exudate

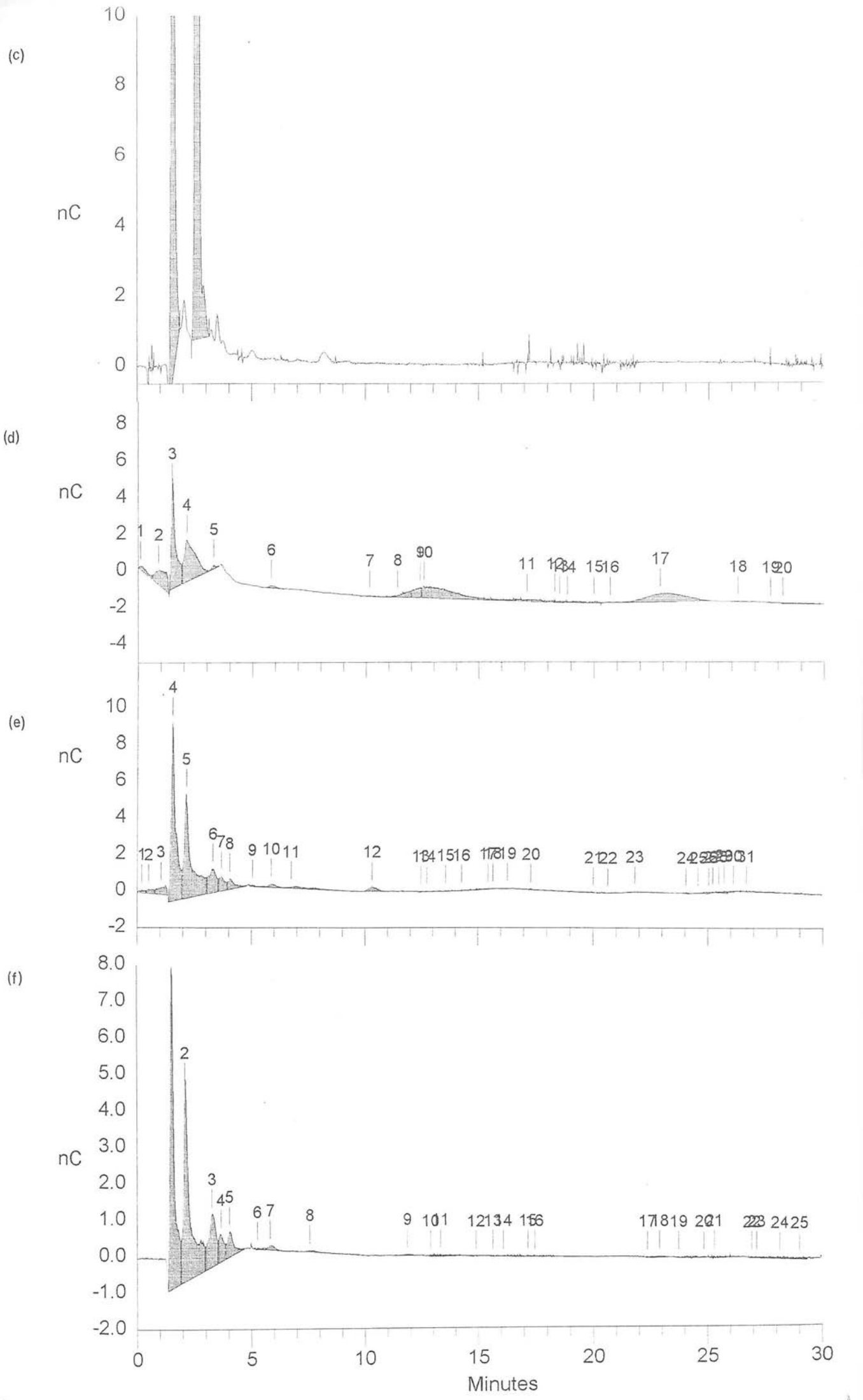
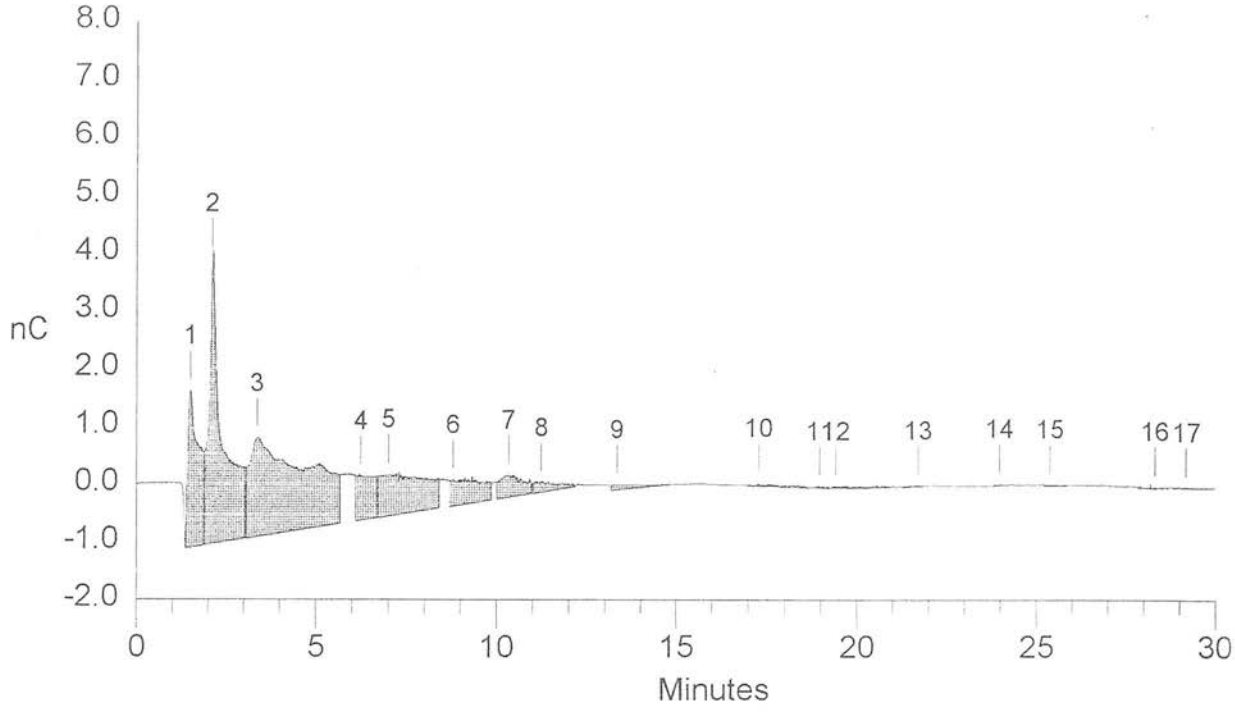

(c)
nC
10
8
6
4
2
0
(d)
nC
8
6
4
2
0
-2
-4
1
2
3
4
5
6
7
8
9
10
11
12
15
16
17
18
19
20
(e)
nC
10
8
6
4
2
0
-2
1
2
3
4
5
6
7
8
9
10
11
12
20
23
(f)
nC
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
0
5
10
15
20
25
30
Minutes

Figure 3.10: Carbohydrate profiles of (g) 50:50 host:non-host root exudate
(h) autoclaved 50:50 host:non-host exudate

(g)


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
nC
1
2
3
4 5
6
7
8
9
10
11 12
13
14
15
16 17
0
5
10
15
20
25
30
Minutes

(h)

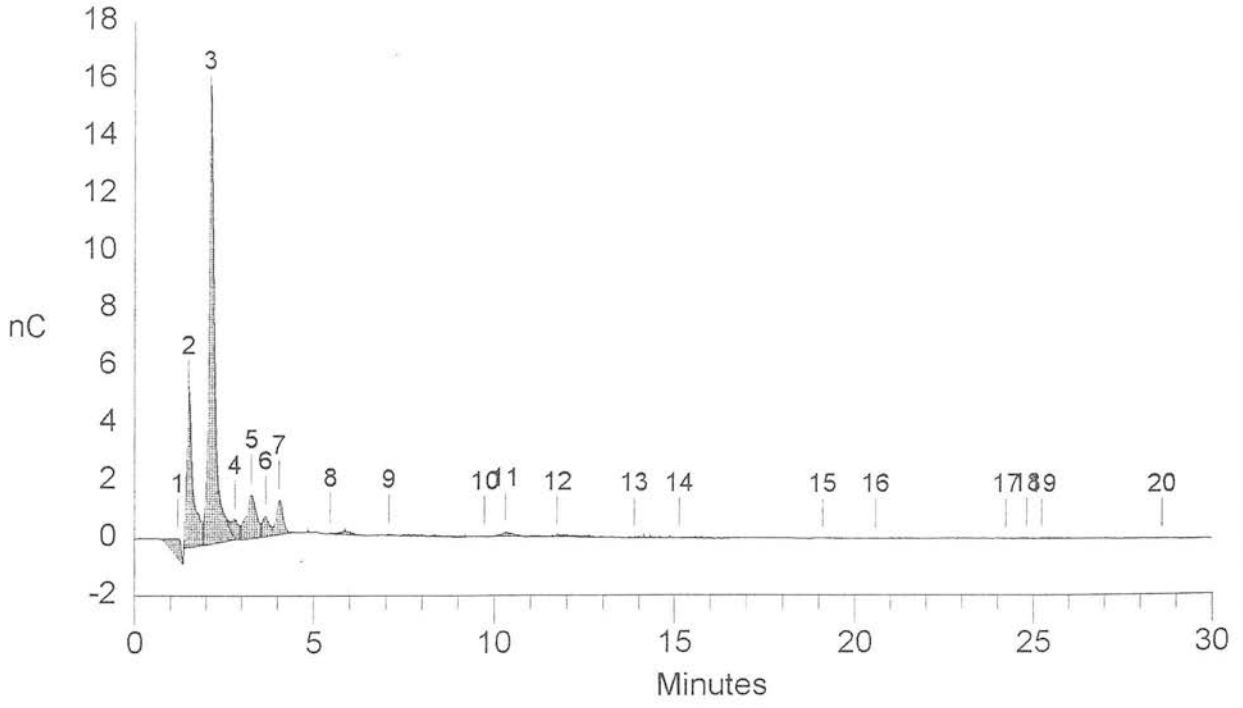
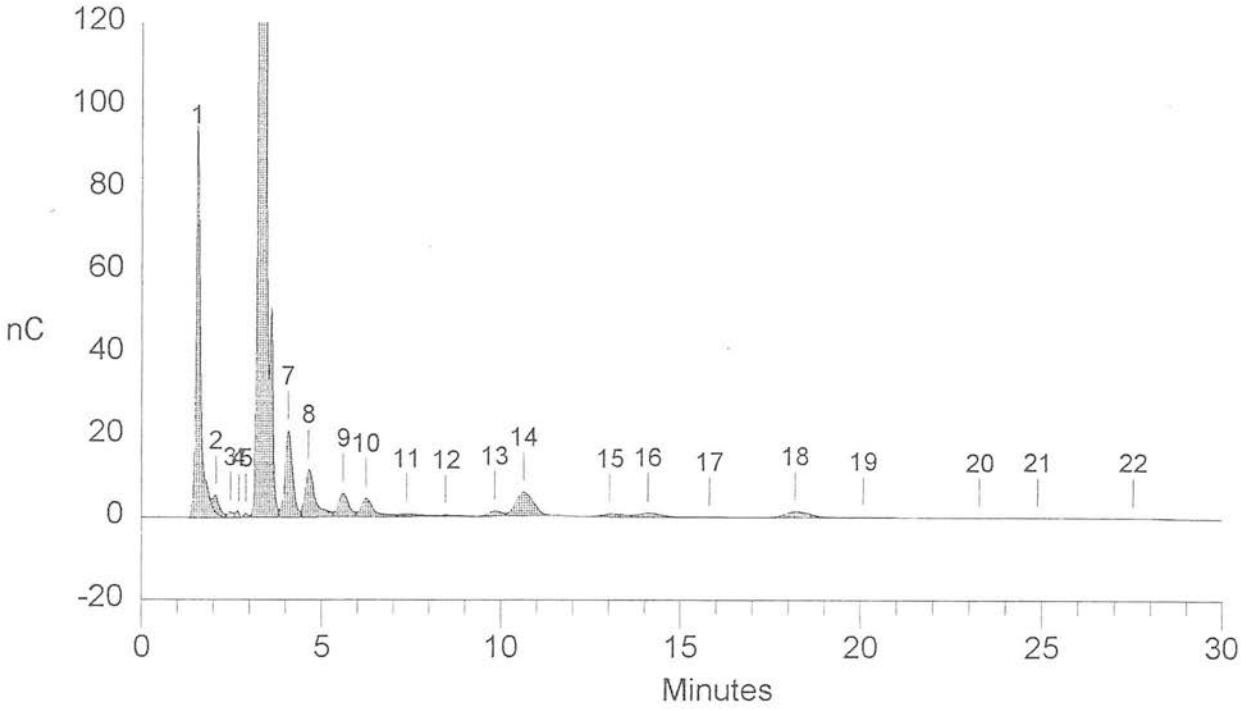
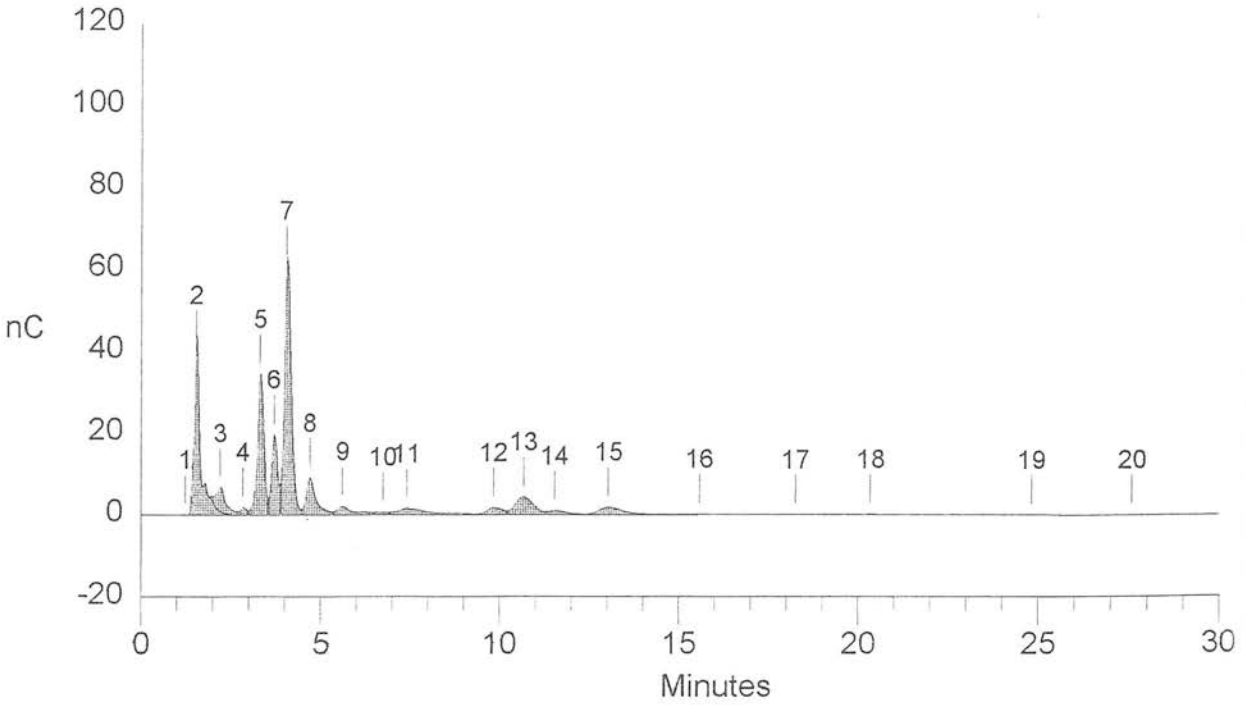

18
16
14
12
10
8
6
4
2
0
-2
nC
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0
5
10
15
20
25
30
Minutes

Figure 3.10: Carbohydrate profiles of (i) non-colonised host root extract
(j) colonised host root extract

(i)


120
100
80
60
40
20
0
-20
nC
1
2
3
4
5
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
0
5
10
15
20
25
30
Minutes

(j)


120
100
80
60
40
20
0
-20
nC
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0
5
10
15
20
25
30
Minutes

glucose (Figure 3.10d). The major peaks eluted within the first 5 minutes. Other peaks were negligible.

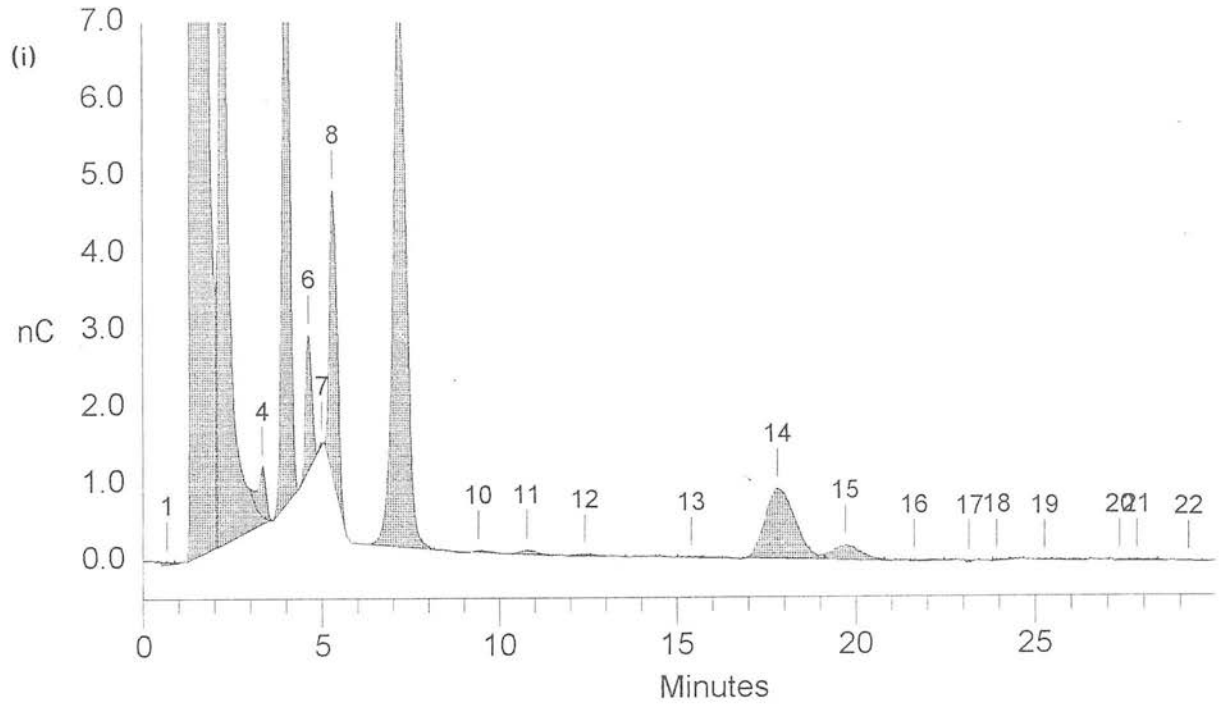
Heat treatment of the host root exudate by autoclaving increased the number of recorded compounds eluting to 31, although peaks for many of these were of negligible size. All major peaks eluted within the first 5 minutes. Glucose, fructose and sucrose were all present. Analysis of heat treated non-host root exudate indicated that the treatment made little difference to the number of compounds present. Again, all the major peaks occurred within the first 5 minutes (Figures 3.10e & f). The slight increase in number of compounds present may indicate that heat treatment was splitting compounds. Compounds that were present in both untreated and heat treated samples were evidently heat stable. A 50:50 mixture of host and non-host exudates did not appear to represent the sum of the compounds found in the individual exudates. The trace appeared similar to that of the non-host rather than the host exudate (Figure 3.10g). Autoclaving the mixed exudate sample increased the number of major peaks eluting within the first 5 minutes (Figure 3.10h).

The non-colonised host root extract produced a profile in which 22 peaks eluted within 30 minutes. This showed the presence of a considerably greater number of compounds than in the exudate, and peak height indicated the occurrence of these at higher concentrations. The major peaks eluted within the first 7 minutes, including glucose, fructose and sucrose (Figure 3.10i). The colonised host root extract contained a similar number of compounds and produced a similar profile, although with different relative quantities of compounds (Figure 3.10j). Differences occurred within the 3-5 minute zone.

Broad similarities were observed between all the samples analysed, all of which contained compounds which eluted at approximately 1.5, 2.1 and 3.3 minutes. It was only possible to identify compounds for which internal standards were available.

Elution through the cation exchange columns resulted in a large decrease in the number of compounds recorded in the non-host exudate, particularly in the range 4-7 minutes. The number of eluted compounds was decreased from 22 to 12 (Figure 3.11a). The root extracts showed little response to cation exchange treatment. Non-colonised host root extracts showed a decrease from 23 to 20 compounds after cation exchange, but profiles remained similar (Figure 3.11b). The colonised host root extract had the same number of compounds after cation exchange treatment (Figure 3.11c). Thus the effectiveness of cation exchange columns was greater with the exudate than with the extracts.

Figure 3.11a: Carbohydrate profiles of non-host root exudate (i) raw sample
(ii) after cation exchange


(i)
7.0
6.0
5.0
4.0
nC 3.0
2.0
1.0
0.0
1
4
6
7
8
10
11
12
13
14
15
16
17
18
19
20
21
22
0
5
10
15
20
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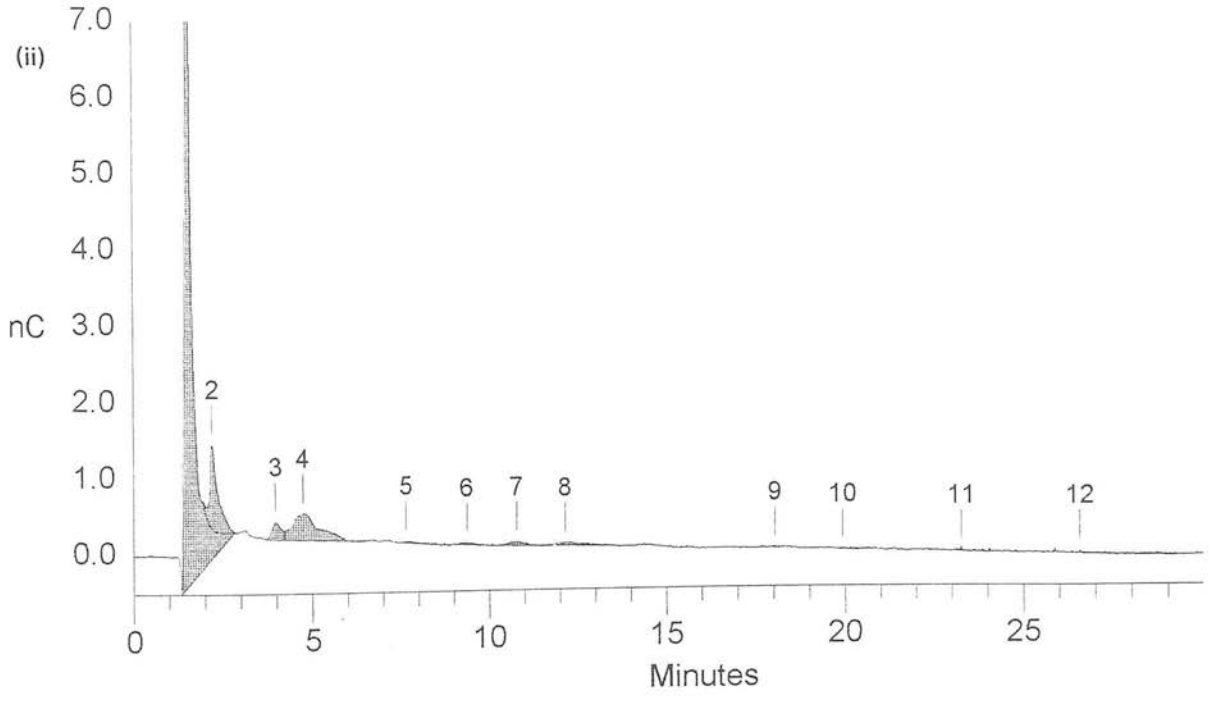
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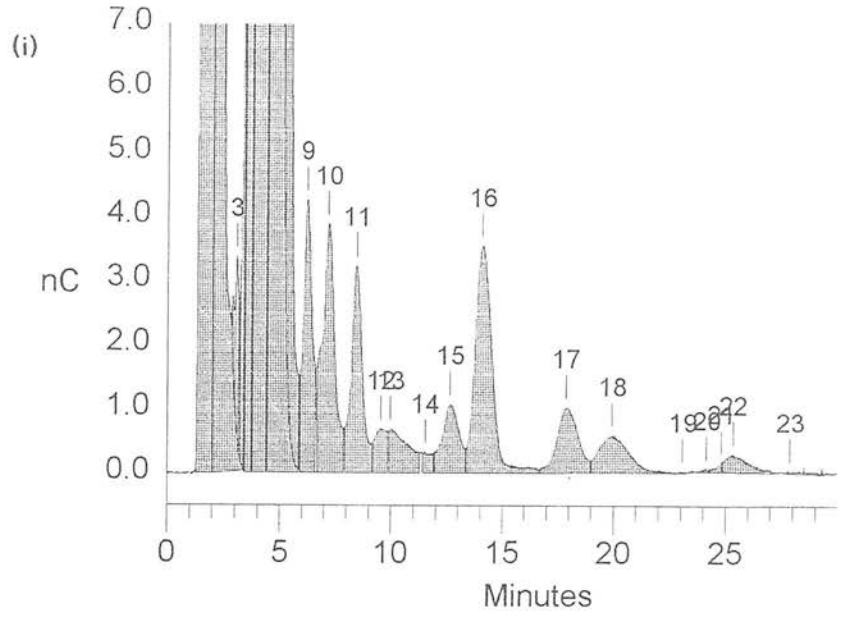
Figure 3.11b: Carbohydrate profiles of non-colonised host root extract (i) raw sample
(ii) after cation exchange

(i)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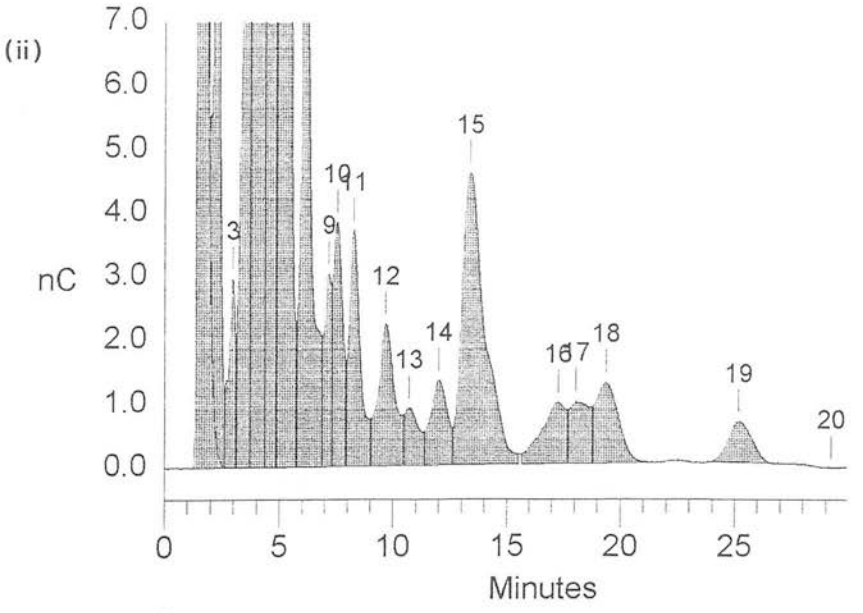
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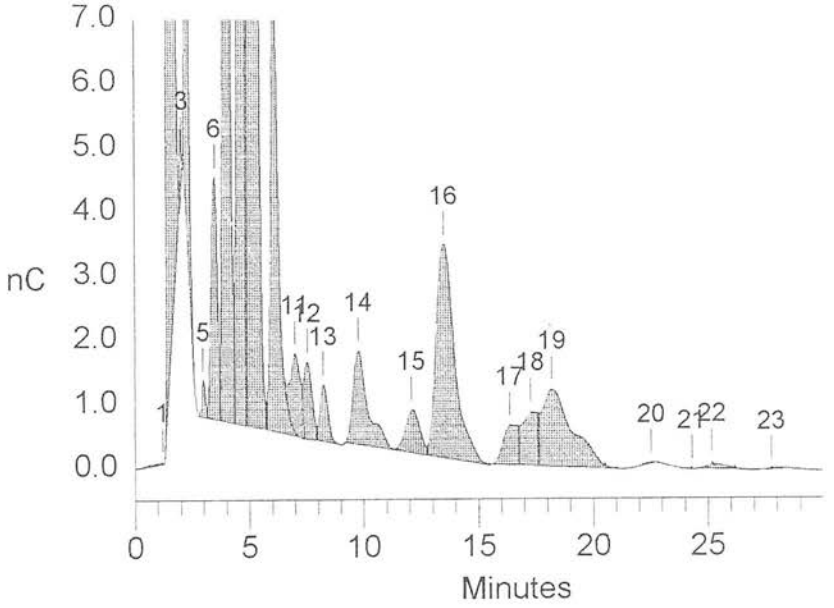
Figure 3.11c: Carbohydrate profiles of colonised host root extract (i) raw sample
(ii) after cation exchange

(i)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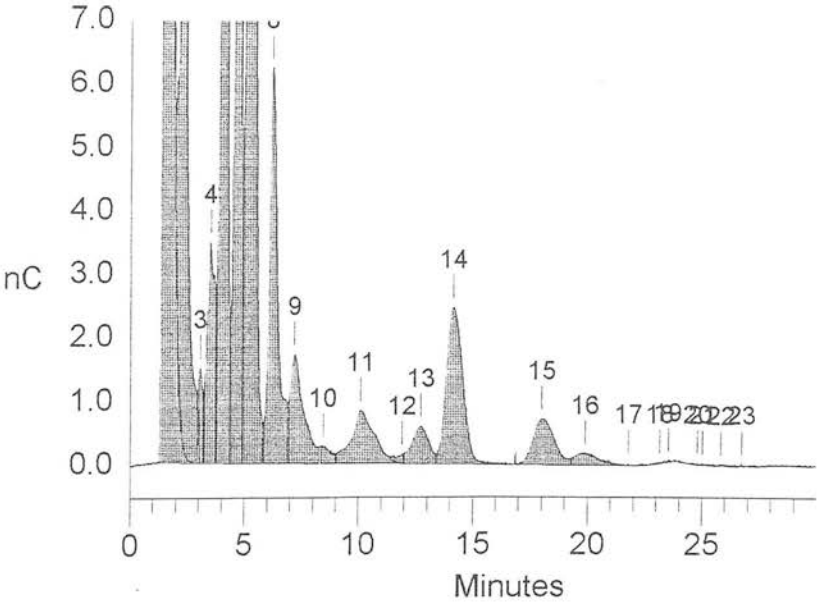
(ii)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3.7.5 Hyphal Branching and Fractal Analysis

The program Fungus (Delas *et al.*, 1996; Section 3.5.5) was run on sections of mycelial images captured via the Q600 (Section 3.3.4.2) and converted to .BMP format. Hyphal branching results exhibited a high degree of variability, and statistical analysis of data was therefore carried out using non-parametric tests (Krukall-Wallis and Mann-Whitney tests; Minitab for Windows Version 10, Minitab Inc., USA). Due to the nature of fractal values, which are restricted to between 0 and 2, data sets cannot vary beyond a limited range. FD data were therefore analysed using ANOVA (Minitab for Windows Version 10, Minitab Inc., USA).

Data from the first experiment examining the effect of placed plugs of agarose impregnated with host exudate on hyphal growth indicated that the degree of branching was reduced in the presence of host exudates. This difference was not significant (Mann-Whitney test p=0.312; Table 3.11). However, the calculated value of FD for hyphal growth in the presence of host exudates was significantly less than in the control (ANOVA p=0.002; Table 3.11). The closer to 1 a value of FD lies, the more linear is the object measured, thus it appeared that the presence of the host exudate was influencing the hyphal growth pattern to a significant extent by producing a more linear growth form. Visual observation did not suggest that this growth response had any directional influence on hyphal growth, but rather an overall effect on the fungal mycelium.

Table 3.11 Fractal dimension (FD) and hyphal branching of regrowth hyphae from AMF colonised root pieces in control experiments and in the presence of host root exudates

Figures are means of 4 replicates

Treatment	Time (d)	FD	No. branches per mm hyphal length
Control	7	1.4^{a}	0.81^{a}
Host Exudate	7	1.0^{b}	0.56^{a}

Within each column figures with the same letter for each data set are not significantly different

In the follow up experiment to this, in which host and non-host exudates and the two flavonoids hesperetin and naringenin were tested in a homogeneous environment, no significant differences were found in either branching (Kruskall-Wallis p=0.279; Table 3.12) or FD (ANOVA p=0.474; Table 3.12). Trends in branching values indicated possible, but non-significant effects of non-host exudates in

decreasing branching, and of host exudates and hesperetin in increasing branching (Table 3.12).

Experiments examining the effects of plant root extracts showed more pronounced responses. The cucumber (host) root extract did not affect hyphal branching. Results appeared to indicate a decrease in the number of branches per mm, but differences were not significant (Mann-Whitney test p=0.244; Table 3.13). In contrast, a significant increase in FD value was observed in the presence of the host root extract, indicating a less linear growth form and more uniform distribution of the mycelium (ANOVA p=0.025; Table 3.13). The non-host root extract significantly increased both the number of branches (Mann-Whitney test p=0.008; Table 3.13) and the value of FD (ANOVA p=0.014; Table 3.13). The colonised host root extract significantly increased the degree of hyphal branching (Mann-Whitney test p=0.0003; Table 3.13) but had no effect on FD (ANOVA p=0.227; Table 3.13) indicating similarities in hyphal distribution within the mycelium despite differences in mycelial complexity.

Table 3.12 Fractal dimension (FD) and hyphal branching of regrowth hyphae from AMF colonised root pieces in the presence of host and non-host exudates and the plant flavonoids naringenin and hesperetin

Figures are means of 4 to 9 replicates

Treatment	Time	FD	No. branches per mm hyphal length
Control	7	1.1[a]	0.54[a]
Host Exudate	7	1.0[a]	0.74[a]
Non-host Exudate	7	1.0[a]	0.30[a]
Hesperetin	7	1.1[a]	0.95[a]
Naringenin	7	1.0[a]	0.59[a]

Within each column figures with the same letter within each data set are not significantly different

Few differences in hyphal branching were evident as a result of heat treatment of host and non-host exudates (Table 3.14). Kruskall-Wallis analysis of results gave a p value of p=0.003, indicating that some significant differences between treatments had occurred. However, application of the simultaneous rank test showed that the only significant difference occurred between the cucumber exudate and the autoclaved oilseed rape exudate and autoclaved control. In both cases, branching with cucumber exudates was significantly greater (Table 3.14).

Table 3.13 Fractal dimension (FD) and hyphal branching of regrowth hyphae from AMF colonised root pieces in the presence of host, non-host and colonised host root extracts

Figures are means of 8 to 16 replicates

Treatment	Time	FD	No. branches per mm hyphal length
Control	7	0.8[a]	1.37[a]
H Extract	7	1.1[b]	0.73[a]
Control	7	1.5[a]	0.36[a]
NH Extract	7	1.6[b]	0.62[b]
Control	7	1.4[a]	0.77[a]
Colonised Extract	7	1.5[a]	2.09[b]

Within each column figures with the same letter for each data set are not significantly different
H Extract = Host plant root extract
NH Extract = Non-host plant root extract
Colonised Extract = Colonised host plant root extract

Table 3.14 Fractal dimension (FD) and hyphal branching of regrowth hyphae from AMF colonised root pieces in the presence of host and non-host root exudates ± heat treatment by autoclaving

Figures are means of 4 to 8 replicates

Treatment	Time (d)	FD	No. of branches per mm hyphal length
Control	2	1.1[a]	1.42
HT Control	2	1.0[a]	0.73[b]
H Exudate	2	1.2[a]	3.71[a]
HT H Exudate	2	1.2[a]	1.36
N-H Exudate	2	1.2[a]	1.31
HT N-H Exudate	2	1.1[a]	0.69[b]

For FD data , figures with the same letter are not significantly different
For hyphal branching data, differences occurred only between values indicated by a letter. No other treatments showed significant differences
H Exudate = Host plant root exudate
N-H Exudate = Non-host plant root exudate
HT = heat treated

The test indicated that heat treatment did not significantly influence the effect of either the cucumber or oilseed rape exudates on hyphal branching, or the effects of these compared with each other. There was no significant difference between the cucumber and oilseed rape exudates, or between either of these and the control. No significant differences were apparent in FD data (ANOVA p=0.311; Table 3.14). These data, as with length data obtained from the same experiments (Section 3.7.2.3; Table 3.8), indicate that heat treatment neither deactivates active compounds nor splits these to produce additional bioactivity.

3.7.6 Statistical Analysis

For experiments in which length measurements were collected over time, mean hyphal growth with time was plotted to allow growth rate comparisons to be made. Comparison of treatment means indicated effects largely similar to those observed from total length data.

Thus, hyphal growth rate in the presence of host root exudates was considerably lower than in the control (Figure 3.13). Similarly, growth rate plots showed that there was no apparent effect of either host or non-host exudates when added seven days after the start of the experiment (Figure 3.14). The apparent increase in growth rate observed in the presence of the host exudates at t = 10 is most likely to be accounted for by replicate variability (Appendix 3: Figure A3.2).

In contrast, growth rate data plots for the experiment comparing the effects of host and non-host exudates and the flavonoids hesperetin and naringenin appeared to show differences in results compared with length data. Previous analysis of total length data indicated that non-host exudates and naringenin were inhibitory to hyphal growth. The growth rate plots showed no effects of these treatments, but indicated that the host exudates were increasing hyphal growth rate (Figure 3.15). However, this is largely attributable to the effect of outlying values in a single replicate (Appendix 3: Figure A3.3).

Problems associated with this method of analysis are discussed in Section 3.9.

3.8 Conclusion: Summary of Experimental Results

This section of the work started with the development of a system for *in vitro* growth of AM fungal hyphae. A number of techniques were tested. Of these, the use of dialysis membranes as the growth substrate provided the optimum system. Desiccation of the membranes was prevented by their direct placement onto molecular grade agarose gel in sealed Petri dishes.

Experiments were subsequently carried out to ensure that agarose gel provided an inert carrier for test substances. This was found to be the case, and thus a system was developed which allowed impregnation of agarose with a given test compound presented as either a point source, or as a homogeneous substrate.

Experiments were then carried out to test the effects of a number of plant-derived substances on AM hyphal growth. The timing of application of test substances was found to be important in hyphal response. Compounds found to influence hyphal growth when applied at the onset of growth had no effect when applied after growth was already established.

Summary: test substance applied at the start of growth as a point source

Host Root Exudates

No directional response was observed. Hyphal growth, measured as length, was depressed in the presence of the host root exudates. This was attributed to a decrease in the fractal dimension (FD) of the mycelium. Hyphal branching was depressed, but not significantly so.

Summary: test substances applied at the start of growth as homogeneous substrates

Host Root Exudates

No significant effect on hyphal growth, measured as length, was observed, nor were there any significant effects on either hyphal branching or FD.

Non-Host Root Exudates

Hyphal growth, measured as length, was significantly decreased. There were no significant effects on either hyphal branching or FD.

Naringenin

Hyphal growth, measured as length, was significantly decreased. There were no significant effects on either hyphal branching or FD.

Hesperetin

There was no significant effect on hyphal growth, measured as length, nor were there any significant effects on either hyphal branching or FD.

Host Root Extracts

There were no significant effects on hyphal growth, measured as length, or on hyphal branching. There was a significant increase in FD.

Non-Host Root Extracts

There was no significant effect on hyphal growth, measured as length. There were significant increases in both hyphal branching and FD.

Colonised Host Root Extracts

There were significant increases in hyphal growth, measured as length, and in hyphal branching. There was no significant effect on FD.

Heat Treated Exudates

Heat treatment of exudates did not influence hyphal growth, measured as length, nor did it have any real effects on either hyphal branching or FD.

Molecular Fractions of Exudates

Analysis of fractions for each test substance

The >10K fraction did not significantly effect hyphal length.
The <3K and 3-10K fractions were biologically active in terms of effect on hyphal length.
The 3-10K fraction appeared to be the most active fraction.
The >10K fraction appeared to be the least active fraction.

Analysis of fraction within test substance

Significant effects occurred between fractions in the control, host root exudate, non-host root exudate and colonised host root extract.

Biochemical Analysis

Protein and Amino-Acid Analysis

All root exudates and extracts tested contained detectable levels of protein.
Host and non-host root exudates had similar amino-acid profiles.
Root extracts contained more amino-acids than exudates, and in greater quantities.

Host root extracts contained more amino-acids than non-host root extracts.
Host root extracts contained more amino-acids than colonised host root extracts.

Carbohydrate Analysis

Host root exudates contained fewer compounds than non-host root exudates.
A 50:50 mixture of the two did not appear as the sum of the individuals, but was similar to the non-host rather than the host exudate.
Host root extracts contained more compounds than host root exudates, and these were present in greater quantities.
Colonised host root extracts had similar profiles to the non-colonised host root extracts, but different relative quantities of the compounds were present.

Summary of Main Points Achieved

(1) Identification of a suitable system for *in vitro* growth of AMF hyphae
(2) Identification of a suitable method for, and timing of, application of test compounds
(3) Tests on plant-derived factors and examination of their effects on hyphal growth, measured as length, and morphology, measured as hyphal branching and FD
(4) Crude identification of compounds:
heat stability
molecular sizing
biochemical analysis

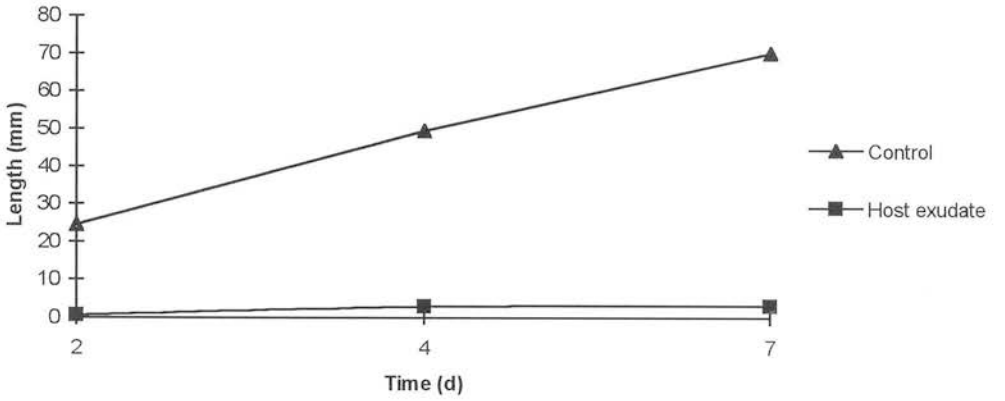

Figure 3.13: Hyphal growth rates (mm/day) in the presence of host root exudates and compared with a control

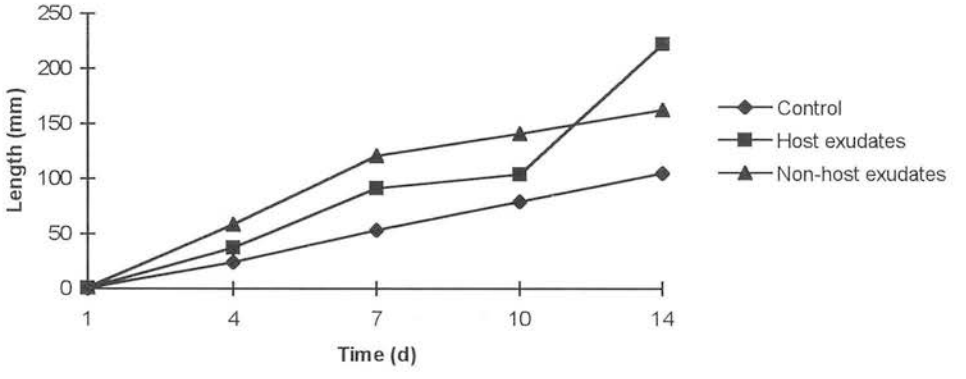

Figure 3.14: Hyphal growth rates (mm/day) in the presence of host and non-host exudates added at t=7

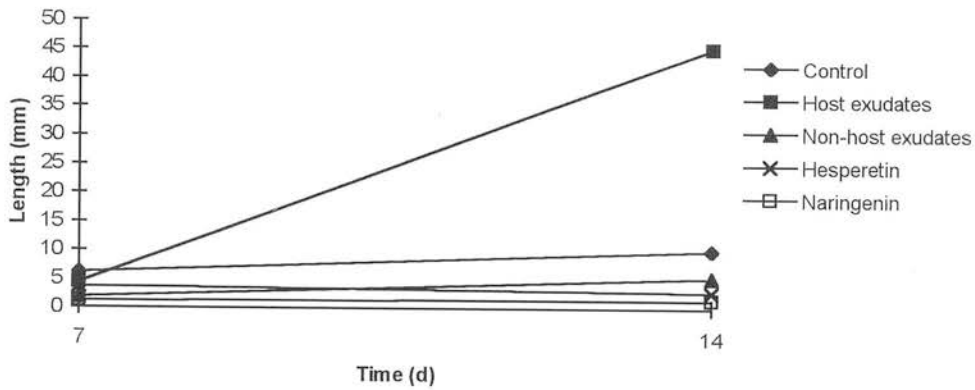

Figure 3.15: Hyphal growth rates (mm/day) in the presence of host and non-host exudates and plant flavonoids

3.9 Discussion

Recent mycorrhizal research has concentrated increasingly on the physiological aspects of the symbiosis and on the functioning and regulation of the plant-fungal association, but there is still a considerable lack of knowledge in this field (Beyrle, 1995) and information remains to a great extent speculative. The state of knowledge specifically concerning the pre-symbiotic phase of AMF is also limited. While a vast amount of research has been carried out on the intra-radical phase of the symbiosis, and on fungal-host contact and plant cellular responses, the study of extra-radical growth has been hampered by the inability to grow AMF in pure culture and the lack of satisfactory protocols for ultra-structural and morphological observations (Bonfante & Bianciotto, 1995). Of all the mycorrhizal structures, the external mycelium, which is arguably the most important in host plant nutrition, is the most neglected and both function and presence are often ignored (Read, 1992). This is largely because it is the most difficult part of the system to examine and manipulate experimentally.

In this study it was decided to concentrate on the effects of plant-derived influences on AMF. It is recognised that, as described in Section 3.2.3, the soil environment plays a major, and largely unexplored, role in the AMF symbiosis. However, there has already been considerable research interest in plant-related effects on spore germination and the growth of germ tube hyphae, and this study aimed to examine differences that may occur in the extra-radical phase, and to discuss these within the context both of existing work, and the AMF life cycle.

Development of Experimental Methodologies

Due both to the natural growth form of AMF as symbiotic organisms which cannot as yet undergo continuous culture in the absence of the host plant, and to the opacity of the soil medium itself, the development of a technique suited to both hyphal growth and visualisation was an important and necessary phase of the project. The experimental technique which was finally used was evolved through a series of stages, with the ultimate aim of developing a system that allowed non-destructive observation of the spatio-temporal effects of a number of plant derived compounds on extra-radical hyphae of AMF. The use of dialysis membrane overlying high purity agarose gel provided a system capable of fulfilling both of these criteria. Problems encountered with this method, largely relating to the degree of manual input required in the generation of final data values, are discussed below.

The calculation of concentrations of bioactive substances is difficult, and therefore selection of concentrations for *in vitro* use has often been arbitrary (Rovira, 1969). While it was recognised in this study that experimental conditions differed physically and nutritionally from natural rhizosphere conditions, the *in vitro* assessment of plant derived influences on AMF attempted to use plant root exudates at "natural" concentrations. This was initially achieved by allowing plantlets to grow in the agarose medium and then carefully removing them, assuming uniformity of exudate diffusion throughout the medium (as in eg. Gianinazzi-Pearson *et al.*, 1989), and latterly by collection of root exudates into water which was then used in making up the agarose medium.

The aims of the project differed from similar work carried out by other authors (particularly Giovannetti *et al.*, 1993 a & b and 1996) primarily in the use of external hyphae of AMF in place of spores and germ tube hyphae, but also in the use of non-destructive visualisation techniques which were developed to facilitate observation of the development of the AMF mycelium over time. Most knowledge gained to date has been achieved through the use of destructive sampling and staining techniques. Giovannetti *et al.* (1993b) used millipore membrane sandwiches for colonisation of plantlets with spores of AMF under sterile conditions in order to assay differential hyphal morphogenesis elicited by factors associated with host plant roots. These membranes were stained destructively with trypan blue after removal of the plantlets to enable microscopic assessment of hyphal growth. Subsequent modification of this method used spores germinated on membranes placed on water agar, and then transferred on the membranes to the roots of sterile plants in Petri dishes. Fungal responses were again assessed microscopically after destructive staining (Giovannetti *et al.*, 1996).

The work described here provides complementary and additional information on extra-radical hyphal growth that may help to highlight the differing roles of the spore and external hyphae in the AM symbiosis, and provide information concerning the environmental responses of different phases of the AMF life cycle.

Although a method of producing high contrast mycelial maps was ultimately developed for AMF grown in this way, the technique remained extremely time consuming. This was largely due to two factors. Firstly, although hyphae observed by light microscopy on a membrane substrate could be relatively easily visualised on-screen via a video-link, contrast between hyphae and background was often poor, and despite grey level processing (Section 3.3.4.3), processed images were not of a quality high enough to enable direct detection and measurement on screen. This necessitated the additional steps of printing out the images, comparing printed images

with the on-screen image and hand editing where necessary, and tracing the printed images onto acetate film for image analysis.

Secondly, because hyphal growth almost without exception exceeded a single microscopic field of view when using a magnification sufficient to capture details of the mycelium, in many cases the mycelium of a single replicate for any treatment required up to 50 sub-images to be captured and used to recreate a composite image of the whole. Thus, in addition to initial capturing of images from microscope to image analysis system, the problem was augmented by the need to subsequently print out and edit this number of images, and fit the images together to recreate the whole mycelium prior to making the final tracing onto acetate for use in measurement of length. Although this method remained the best of those developed and tested for the observation of the mycelium of AMF over time, the problems encountered indicate a clear need for further investigation of semi-automatic image analysis systems able to deal with the type of images involved, and thus cut down on image preparation time.

As discussed in section 3.1.2.1, increased image complexity increases the difficulty of creating automated image analysis programs that are capable of dealing with the images. It is therefore unlikely that any program will be developed which does not require some degree of user intervention. The full potential of image analysis in the characterisation of the morphology of filamentous microorganisms will probably be achieved only by automation using application specific software to replace general purpose programs (Packer & Thomas, 1990). Work is currently underway to determine a number of semi-automatic techniques for segmenting (extraction of features from an image) but the success of such a program is likely to be dictated by the complexity of the image presented to it. The computer can aid a purely manual approach because of its "number crunching" abilities, but the user is required to help in the communication process because of the inability of the computer to yield satisfactory results on its own.

These semi-automatic methods are based on one of two approaches. The first relies on a "computer-user" technique, in which the user is required to interact with an initial computer solution. The method requires a battery of image analysis techniques because of the difficulty of "explaining" events to the computer. The second is based on "user-computer" analysis, in which the computer refines initial user inputs or solutions. The second method has the advantage that the computer already has a guideline from which to work. With images such as the AMF mycelium it is likely that, at best, a purpose written program could make it possible to cut down the degree of manual tracing required by automatically creating a relatively accurate skeletonised representation of the hyphae, and giving the user the option of editing in

any sections which were inaccurately imaged. Such a program may also include an option allowing the removal of any artefacts in the image that arise from inaccurate skeletonisation (Iain Inglis, pers. comm). The difficulties involved are indicative of the problems that arise in totally automated programming for complex image analysis, in which a gain in speed of image processing is likely to be at the cost of accuracy (Packer & Thomas, 1990).

Experimental Procedures

Along with assessing the general growth responses of extra-radical hyphae of AMF to applied environmental stimuli, one of the initial aims of the project was to examine any directional responses that may occur to heterogeneously applied stimuli. Given the widespread occurrence of chemo-tactic and -tropic responses in nature (Section 3.2.2) and the obligate nature of the symbiosis, it seemed possible that AMF hyphae may be directionally responsive to exudates of their host and non-host plants, or to constituents of these. While previous authors have found many and varied effects of plant products on AMF, in general there have been few references to chemotropism, and these have referred to hyphal growth from spores rather than to extra-radical hyphae (Gianinazzi-Pearson *et al.*, 1989; Giovannetti *et al.*, 1993b).

Generation of comparative data may serve to highlight potentially important differences between behaviour of AMF hyphae originating from spores, and those from root piece inoculum. Studies of other species of fungi have given rise to anomalies which suggest the need to consider fungal responses to environmental factors at different stages of their development from spores. Vegetative hyphae of *Pythium aphanidermatum* (Edson) Fitzp. (CBS 634.70) and *P. graminicola* Subramanian (CBS 327.62), for example, were observed not to respond tropically to nutrients despite the occurrence of chemo-responses with their germ tubes (Mitchell & Deacon, 1986). AMF produce spores in the soil which are capable of germination in the absence of host roots. This indicates the evolution of an efficient strategy for location of specific hosts (Giovannetti *et al.*, 1996), and promotion of hyphal growth from spores due to the effects of host plant root exudates is well documented (Bécard & Piché, 1989b; Nair *et al.*, 1991; Giovannetti *et al.*, 1993a). Changes in the morphology of AM fungal hyphae have also been observed by previous authors in the presence of host root exudates (Mosse & Hepper, 1975; Powell, 1976; Mosse, 1988) and it has been shown that host derived signals are the cues to which AMF respond in the early stages of infection, through the initiation of differential branching patterns prior to the formation of infection structures (Giovannetti *et al.*, 1993b). Hyphae from root segments may exhibit fewer deficiencies in both nutrients and hormones than

those from spores (Powell, 1976) which have limited reserves (Mosse & Hepper, 1975), and this may account for a less pronounced response to root exudates (Powell, 1976).

The use of mycorrhizal root pieces rather than spores in this study not only opened up the possibility of increasing understanding of the growth and environmental responses of AMF, but also raised additional difficulties in terms of methodologies. Spores are most frequently used in AMF research because of their relative ease of identification, extraction, sterilisation and germinability *in vitro*. In addition, despite the possibility that they may undergo multiple germinations which can cause variability in germination time and subsequent hyphal growth (eg. Mosse, 1988) they provide a more uniform starting point for experimental use. Hyphae regenerated from root pieces are similarly recognised as potential starting material for experimental use (eg. Williams 1984, 1990), but results highlighted the problem of variability of hyphal growth *in vitro* and raised the possibility of temporal variation occurring in root piece inoculum between different experiments. This problem was partially overcome by the careful selection of mycorrhizal root pieces from the same root of a mycorrhizal host plant, and by the use of an individual control for each experiment that was carried out. Despite these precautions, considerable variability still occurred. The obvious method of overcoming this problem was to increase replication. This was feasible to a certain extent, beyond which the time consuming nature of the work made it impossible to increase replication further. As the work progressed, the methods used were evolved in an effort to decrease the problems of variability.

The initial aim of examining chemotropic responses in AMF to a number of plant derived compounds was amended in response to difficulties in assessing this parameter. It appeared from the first series of experiments in which stimuli were provided as point sources at distances of 5mm from the root piece inoculum (Section 3.5.1) that no chemotropic responses were evident. Over the years there has been a general assumption that, despite the relatively low density of AMF spores in soil, and the nutritionally obligate nature of the symbiosis, contact between fungus and host results from a sequence of passive events mediated by chance. Koske & Gemma (1992) recently described this assumption as untenable, suggesting that while chance encounters cannot be ignored, other mechanisms may be much more biologically significant. In fact there is little and varied evidence of the mechanism by which contact between the symbionts occurs. Gianinazzi-Pearson *et al.* (1989) found that no directional response to the presence of flavonoids occurred in germ tube hyphae, despite effects of these compounds on spore germination and early hyphal growth. In contrast, Koske (1982) found that unidentified volatile compounds from corn and

bean roots stimulated a chemotropic locational response in aerial germ tube hyphae of *Gigaspora gigantea*. Giovannetti *et al.* (1993b) detected a distinct stage in the early events of AMF infection, occurring prior to appressorium development, which indicated that not only were factors associated with host plant roots recognised as early signals by AMF, but that this recognition triggered both re-orientation in direction of growth, and a change in branching behaviour. Changes in hyphal growth in the presence of host roots are mainly the result of changes in hyphal morphogenesis triggered by root-associated factors, allowing speculation that host root exudates facilitate the location of suitable sites for adhesion and appressorium formation. This identifies chemodifferentiation as the earliest detectable morphological event in the recognition of the host plant by AMF (Giovannetti *et al.*, 1993b). Giovannetti *et al.* (1993b) found that roots of a number of test host plant species were able to elicit hyphal proliferation in *Glomus mosseae*, *Glomus intraradices* and *Glomus 'A6'*. Non-host plant roots had no effect on hyphal growth, reinforcing previous hypotheses that non-host roots lack factors promoting hyphal growth in AMF (Giovannetti *et al.*, 1993a). Despite their status as obligate symbionts, AMF have been observed to show surprisingly little stimulation by host roots in the soil, with neither spore germination nor initial direction of hyphal growth responding to the presence of host roots (Powell, 1976). Despite continued interest in chemo-oriented aspects of the growth of AMF, it was decided that in subsequent experiments only one parameter, that of hyphal growth as measured by length and branching, would be assessed.

It was also noted during experimentation that where the addition of the stimulus was delayed until after hyphal growth was already established, no effects of the applied compounds were apparent (Section 3.7.2.1). This suggested that where hyphal growth was already established prior to application of test compounds, growth responses did not occur, and that hyphae were able to respond only to compounds applied either before the start of growth, or in its early stages. This provides an interesting comparison with the results obtained by Giovannetti *et al.* (1993b) which suggest that AMF hyphae from spores are able to change both direction and form of growth in response to environmental factors in the early stages of growth prior to appressorium formation. The apparent inability observed here of an established AMF mycelium to respond to environmental stimuli by plasticity of growth suggests an inflexibility of growth form at odds with the complex nature of the organism's environment and its relationship with plant root systems. It also raises an interesting comparison with plant root systems themselves, which occupy a similar environment and exhibit a broadly similar growth form, but which while exhibiting basic genetically determined morphological patterns also have a plasticity of form which facilitates

changes in growth and development in response to environmental changes (Merrill *et al.*, 1994). Both are the principle nutrient absorbing organs of their parent organism, and have been observed to show similarities in response to localised sites of nutrient availability (Kimmins & Hawkes, 1978; St. John *et al.*, 1983b). As a result of this observation, in further work to assess the effects of a number of compounds on hyphal growth, the compounds were added at the start of the experiments.

In effect, adapting the methods served to solve one problem while creating another, as by attempting to increase the uniformity of the experimental conditions to decrease variability of results, the sensitivity of the method was decreased. Thus, the method used at the end of the study in the AMF growth bioassays (Section 3.5.3) enabled only a visual assessment of hyphal development using a scoring system based on hyphal coverage of a grid, and did not enable any precise measurements of actual hyphal length or branching characteristics. Obviously, the precise aims of the work must be accommodated by the method chosen, and it was only by modification of the aims that more rapid methods could be developed in this study.

Experimental Results

Rhizosphere organisms are influenced primarily by two major factors, those originating from the plant, and those from the soil. This study chose to concentrate on plant derived influences, and as such the effects of mycorrhizal host and non-host plants, their root extracts, exudates and derivatives of these were studied. Plant root exudates are those substances released into the surrounding medium by healthy and intact plant roots, and provide the earliest communication mechanism in regulation of the mycorrhizal symbiosis. Such communication between the symbiotic partners is an essential process, first encouraging and later permitting the formation of a physical association between different species of organism (Koske & Gemma, 1992). A wide range of compounds can be exuded by a single plant species, some of which are primarily nutritive in function, with a role in growth promotion, while others may trigger a change in the fungus that promotes colonisation. Differential effects of host and non-host plants on mycorrhizal fungi may be manifest without actual root contact (Anderson, 1992).

In the present study, the results of the first experiment assessing the effect of host plant root exudates on AMF hyphal growth from root pieces showed that the host exudates decreased the length of hyphae produced (Section 3.7.1). This result initially appeared surprising, as previous work using AMF spores indicated a positive effect of host root exudates on germination and early hyphal growth (Gianinazzi-Pearson *et al.*, 1989; Giovannetti *et al.*, 1993a). Such differences may arise because,

as discussed earlier, the germ tube and extra-radical hyphae have separate roles in the symbiosis which make it likely that the two sources of inoculum will respond in different ways to environmental influences. The spores have a major role in the initiation of new infections, and as such may be expected to respond positively (and directionally) to the presence of a host plant. In contrast, the extra-radical hyphae of AMF have a more diverse role in the symbiosis, as they may be responsible for either the initiation of infections or the uptake of nutrients. The phenomenon of dimorphism in AMF hyphae has already been recognised. Hyphae may be either predominantly simple in both structure and function with a primary role in infection, or have a more complex branched form that has a largely absorptive role (Friese & Allen, 1991). Thus, differences in response between germ tube and extra-radical hyphae to similar environmental influences may arise from the ability of the extra-radical hyphae to alter growth form according to environment. Visual observation of hyphae incubated in the presence of host root exudates suggested that differences in growth form may be partially responsible for measured differences in length, and this observation led to the development of a method aimed at quantifying differences in the degree of hyphal branching, and in hyphal distribution within the mycelium (measured as FD; Section 3.5.5). This is discussed later. The observed response may be indicative of a change in nutrient status associated with the presence of the host plant, which will be in competition for nutrients within the same soil volume, and to which the hyphae exhibit a recognition response mediated by bioactive compounds in the plant root exudates. This hyphal response is evident as a decrease in length and branching representative of a functional change to the infective state, indicating that extra-radical hyphae may respond to the application of host root exudates by a change in form to the more linear "runner" hyphae characteristic of their infective phase. The presence of a host may thus trigger the colonisation potential of the AMF, enabling the initiation of new infections when in the proximity of a compatible host plant. Conversely, in the absence of a plant (as simulated by the control) the absence of signal molecules and lack of competition would indicate increased resource availability, stimulating increases in hyphal length and branching characteristic of their absorptive function. Thus, the absence of a host plant may trigger the uptake potential of AMF.

Further experimentation which repeated the application of host exudates to AMF colonised root pieces also examined the influence of non-host exudates and of plant flavonoids on hyphal growth (Section 3.5.2.1). Results of this work served again to highlight the problems encountered with variability of hyphal growth from root pieces, and the differential responses occurring between germ tube and extra-radical hyphae. Results showed that neither the host exudates nor the flavonoid hesperetin

had any effect on hyphal length after 14 days incubation, while both non-host exudates and the flavonoid naringenin significantly decreased hyphal growth after seven days. After 14 days, only the naringenin was still eliciting a significant response. This supports the earlier suggestion that hyphae may be able to repond more readily to test environmental influences applied during the early stages of growth, and that reponses decrease as growth progresses. In this case, hyphal length was not depressed in the presence of host exudates as had been observed in earlier work. However, neither was it stimulated.

The initial depression of, and subsequent lack of effect on, hyphal growth observed in the presence of non-host exudates in this experiment supports previous authors in their assertions that non-host roots lack factors capable of hyphal growth promotion (Gianinazzi *et al.*, 1989; Bécard & Piché, 1990; Giovannetti *et al.*, 1993a). Giovannetti *et al.* (1993a) suggested that non-host plant inhibition of spore germination and early hyphal growth, manifest as a hindrance to hyphal attachment and fungal recognition of hosts, may alternatively result from shoot-produced inhibitory factors or induced changes to the rhizosphere pH which hinder initial hyphal approaches to roots. Depression of growth may alternatively be due to the presence of fungi-toxic substances, although the fact that a 50:50 mixture of non-host to host roots did not significantly affect hyphal growth makes this unlikely here.

In the presence of a non-host the hyphae are unlikely to change their form to an infective type hyphae which may otherwise have accounted for decreases in growth. However, it is possible that hyphae in the presence of a non-host recognise the likelihood of competition for nutrients without the benefits in carbohydrate supply that occur in the formation of a successful symbiosis with a host plant. Thus a decrease in growth may represent the formation of linear hyphae which are able to cover a large distance with minimum use of resources in the search for either a host plant or an area of soil free of competition from a non-host plant for exploitation of nutrients. Alternatively, this response may be indicative of non-host recognition due to the production of antagonistic compounds in non-host root exudates. Despite observed decreases in hyphal growth, non-host exudates were still able to support a limited amount of growth. This supports previous evidence obtained by Ocampo *et al.* (1980) that AMF hyphae are able to grow in the presence of non-host plants, possibly deriving some benefit from the non-host plant root system, particularly if host plants are also present. Consequently, the presence of a non-host may be more favourable to AMF development in soil than the absence of either a host or a non-host plant. In this case, the amount of root exudation may be important through its

effects on nutrient availability, which may make the presence of the non-host more tolerable (Koske & Gemma, 1992).

That a dialogue exists between the partners of the symbiosis, allowing the unambiguous selection of host and symbiont, is now well established. Detailed work concerning host and non-host induced responses in the growth of AMF from spores has recently been carried out by Giovannetti *et al.* (1994). Results of this work indicate that AMF are able to distinguish their hosts from all other plant species, including both non-mycorrhizal species and hosts of non-arbuscular mycorrhizas. The recognition process operates via root-emitted signals, which provide early cues to the fungus and trigger a switch from the symbiotic to the infective state. Plant defence reactions occur only after this switch has taken place (Giovannetti *et al.*, 1994). All plant species that do not act as AMF hosts have in common the lack of a signal which enables this early recognition of hosts by AMF.

Whether or not a similar communication mechanism exists between different plant species and the external phase of the AMF is less clearly defined by the results of the present work, but early indications suggest that this is a possibility. Just as the perception of the correct chemical signals from host plant roots can promote differential hyphal morphogenesis in germ tube hyphae from a growth pattern that maximises the chance of root contact to a profusely branched state prior to physical interaction with the host (Giovannetti *et al.*, 1994), so may host signals trigger changes in the extra-radical growth form which are able to promote functional morphogenesis in relation to environmental conditions.

Evidence concerning the influence of plant flavonoids on AMF is contradictory. While it has been suggested that they play an important role in spore germination and early hyphal growth (eg. Gianinnazzi-Pearson *et al.*, 1989), their precise role as regulators of the mycorrhizal symbiosis remains in question. It may be that it is the relative effects of different flavonoid compounds that are important, and that ratios of stimulatory to inhibitory compounds define the response (Gianinazzi-Pearson *et al.*, 1989). Low nutrient conditions which are favourable to AMF have been found to favour the accumulation of flavonoid compounds in plant tissues, indicating a potential regulatory role in the AMF symbiosis. However, there are anomalies. Recent work found no direct evidence for any effect of isoflavonoids on plant growth, indicating that observed plant growth stimulation is likely to be mediated by the AMF itself (Siqueira *et al.*, 1991). In addition, some phenolic compounds are known to have allelopathic qualities, and inhibitory effects on root colonisation by AMF have been observed (Pederson *et al.*, 1991). Recent work by Bécard *et al.* (1995) found that flavonoids are not necessary for the establishment of

the mycorrhizal symbiosis, and it was concluded that these compounds are not plant signals in the AM symbiosis. It similarly appears from this study that flavonoids are not signal molecules for extra-radical hyphae of AMF. The results of different authors again highlight the possible differences that exist between biochemical influences on spores and on extra-radical hyphae. Alternatively, it may be that the presence of phenolic compounds in root exudates induce not morphological changes, but changes at the genetic level, inducing gene expression in mycorrhizal hyphae before their arrival at the root surface. Hyphae unable to respond by synthesis of the correct "molecular key" would thus not be recognised as potential symbionts at the host surface (Anderson, 1988). What does seem likely is that successful establishment of the mycorrhizal symbiosis is under the control of a number of independent processes which are highly specific to different phases of its development.

In general, knowledge of the identities of root exudates and cellular metabolites is inadequate. Differences between field, glasshouse and laboratory conditions can cause both qualitative and quantitative differences in plant root exudates and secondary metabolites (Rovira, 1969) and exudation in culture may under-represent that in soil (Koske & Gemma, 1992). There is mounting evidence to suggest an even greater degree of complexity in the plant-fungal relationship. Plants and mycorrhizal fungi appear to have evolved a complex and efficient system of chemical communication to maximise the chance of contact between the two partners, over which both have some degree of control. Thus, although responses of the fungal partner to the presence or absence of host and non-host plants may not always be evident, plant responses to the fungus may be occurring. There are two mechanisms by which young colonisable roots can apparently be induced to approach spores or hyphae. Firstly, stimulation of root branching can occur after contact of roots by germ tubes and/or the development of infection. Secondly, the attraction of root tips to spores can occur after germ tubes have failed to successfully locate roots (Gemma & Koske, 1988).

Neither host nor non-host root extracts had any effect on AMF hyphal growth (Section 3.7.2.2). The difference in response observed between exudates and extracts could be attributable to the fact that the exudate profile of a root is not a simple reflection of the profile of the intact tissue and will not therefore elicit the same response. Major metabolites found in root exudates are not always present in the intact root (Bel-Rhlid *et al.*, 1993). Interestingly, the presence of colonised host root extracts resulted in significant increases in hyphal growth, indicating that the presence of the fungus was altering root composition by the addition of stimulatory factors not otherwise present. AMF-mediated influences on root biochemistry are discussed later.

This result ideally requires comparison with growth effects elicited by the presence of colonised host root exudates. As discussed previously (Section 3.7.2.1), contamination problems prevented these results from being obtained.

Problems encountered with contamination, particularly in efforts to test the effects of colonised host root exudates, raise the question of the use of sterile conditions in mycorrhizal research. As discussed in Section 2.5, sterilisation of root pieces resulted in a decrease in hyphal growth despite considerable efforts to develop a technique capable of removing contaminants without detrimental effects on the AMF. For this reason, the present work was carried out under semi-sterile conditions ie. all Petri dishes, media and membranes were sterilized, but root piece inoculum was not. Studying the effects of root derived compounds on AMF under sterile conditions would undoubtedly have guaranteed that any observed effects were due solely to the test substances and not to the presence of any microbial contaminants. It was, however, observed by Giovannetti *et al.* (1996) that the degree of sterility of an *in vitro* system did not influence the response of AMF to host-derived signals, indicating that it was factors derived directly from roots which elicited the recognition response in AMF. Nevertheless, it remains important to recognise not only that the rhizosphere population may modify biological effects as a result of absorption of some compounds by microorganisms, and excretion of others (Rovira, 1969; Koske & Gemma, 1992), but also that intimate relationships may exist between AMF and rhizobacteria which are likely to be important in bacterial colonisation of plant root systems and which suggest that mycorrhizal systems can in fact consist of a combination of plant, fungal and bacterial cells (Bianciotto *et al.*, 1996a & b).

Limitations of the assay techniques employed *in vitro* cannot be overlooked. Much of the initial research carried out on plant-fungal communication mechanisms has concentrated on the water soluble compounds as the primary focus of attention (Koske & Gemma, 1992), failing to acknowledge the limitations of the inability to separate the effects of these from the effects of volatile compounds, which are now known to be equally important (Bécard & Piché, 1989a & b). It is freely acknowledged that this study also failed to separate the effects of these two classes of compounds, but this was without intention to ignore the relative importance of either. Attempts to discuss underlying reasons for observed biological effects are equally applicable to volatile as to water soluble compounds. In fact, formation of mycorrhizal associations is most likely regulated both by water-soluble and volatile compounds, and by surface-bound recognition molecules. The differing chemical properties of these compounds, and thus their relative capacities to move through the soil, dictate their zone of influence over the symbiosis.

Statistical Analysis

The variability of the data dictated the statistical analysis used, restricting analysis to non-parametric tests which acknowledged the non-normal distribution of the data. Statistical analysis was carried out on final total hyphal length measurements, as this data was available for all the experiments conducted.

Growth rate data has seldom been evaluated for external hyphae of AMF, largely because of problems previously discussed concerning the technical difficulties of studying AMF *in vivo*. In the present work, collection of length measurements over time in a number of experiments allowed mean hyphal growth over time to be plotted, with a view to making growth rate comparisons between treatments. In the interests of carrying out full analysis of the available data, this was attempted, but in fact the variability of the data gave rise to similar problems as had been encountered with final length measurements. This is shown in the replicate plots included in Appendix 3.

Comparison of treatment mean plots in fact showed few differences from the final hyphal length data. Thus, an observed depression of total hyphal growth in the presence of host root exudates was similarly evident in a growth rate plot (Figure 3.13, Section 3.7.6; Table 3.4, Section 3.7.1). Observations of the effects of the control compared with host and non-host exudates added at t=7 days also showed comparable results between the two methods of analysis (Figure 3.14, Section 3.7.6; Table 3.5, Section 3.7.2.1). In contrast, apparent differences occurred in the data comparing host and non-host exudate effects with the flavonoid compounds hesperetin and naringenin (Figure 3.15, Section 3.7.6; Table 3.6, Section 3.7.2.1). For an explanation of this apparent difference in results obtained through different data analysis techniques, the reader is referred to Figure A3.3 (Appendix 3), from which it is evident that variability masks any potential effect.

After discussion with a statistical advisor following this preliminary analysis, it was decided that no growth model could be realistically fitted to the data, and that there was little point in trying to transform the data such that a model could be made to fit.

Exudate Analysis

In studying the effects of plant exudates on specific microorganisms, it is unlikely that ubiquitous sugars, amino acids or organic acids make a significant contribution to growth effects. It is more likely to be the balance of these and/or additional "exotic" compounds which are important (Rovira, 1969). In fact, intact plant roots exude relatively small quantities of organic material (generally $\leq 0.4\%$ of photosynthetic carbon) and exudation of compounds with specific bioactivity is often

quantitatively so low that they are barely detectable by chemical or chromatographic techniques. The chemical characterisation of active factors is made easier by the largescale production of exudates for experimentation. This raises the importance of the use of more sensitive bioassays in assessment of growth responses in soil microorganisms (Rovira, 1969). In this study, the bioassays using AMF showed sensitivities greater than those obtained using chemical methods, in which only minor differences were apparent between traces of the various test compounds.

Polysaccharides associated with root mucilage have been attributed a diversity of biological functions, such as zoospore recognition, and an involvement in the mediation of biological phenomena. These compounds represent a diverse group of secretory products which are poorly characterised in biochemical terms (Ray, Callow & Kennedy, 1988). It has been suggested that root exudate sugar content is important in the contribution of root exudates to the arbuscular mycorrhizal symbiosis (Ocampo *et al.*, 1980; Azcon & Ocampo, 1981; Schwab *et al.*, 1982; Azcon & Ocampo, 1984) and early work implicated root carbohydrate concentrations as regulatory in ectomycorrhizal formation, considering a surplus of soluble sugars to be essential in maintenance of the symbiosis (Björkman, 1942 in Beyrle, 1995).

In this work, carbohydrate analysis indicated the presence of a greater number of compounds in the non-host than in the host exudate (Section 3.7.4.2). This appears to suggest that, contrary to previously discussed results of other authors, differences in AMF hyphal growth in the presence of host and non-host exudates may be due to the production of additional detrimental factors, rather than to the absence of stimulatory compounds. Heat treatment did not influence bioactivities of either host or non-host exudates, indicating that active compounds were heat stable and presenting the possibility that the active fraction may have been part of the carbohydrate rather than the protein fraction of the exudate.

The apparently greater number of compounds observed in the extract compared with the exudate may again be indicative of differences between profiles of intact roots and their exudates. It has been previously observed that the sugar content of wheat root extracts can be much higher than that of their exudates (Azcon & Ocampo, 1981). Alternatively, it is possible that carbohydrate compounds in the exudates were present in quantities too low for detection, but were evident in detectable amounts in the extracts. Similarities between the carbohydrate profiles of colonised and non-colonised host root extracts suggest that the presence of AMF does not trigger changes within root tissue. In contrast, previous authors have indicated that infection by AMF leads to a decrease in both reducing and total sugar content in the root, probably because of utilisation of these metabolites by the fungus

(Azcon & Ocampo, 1981). Similar changes in protein profiles of tissues of mycorrhizal and non-colonised plants have been described by a number of authors (see below).

Without doubt, the most widely used techniques of high resolution chromatography for quantitative and qualitative analysis of individual sugars are gas liquid chromatography (GLC) and HPLC. This study used HPLC, the efficacy of which over GLC has been established in recent years, particularly in terms of speed and simplicity of sample preparation (Folkes & Crane, 1988). The use of electrochemical detection has only been relatively recently applied in carbohydrate analysis. The method works on the basis of the application of a simple potential which causes the reaction products to accumulate on the electrode. The use of a triple pulse overcomes the problem of electrode poisoning, and the oxidation current is measured for the first part of the cycle. Using sodium hydroxide as the eluent, this provides a highly sensitive method of carbohydrate analysis. The advantage of HPLC in sugar determination is that derivisation is generally unnecessary, and sample preparation can be simple - the most straightforward method, as used here, is aqueous extraction. The major use of HPLC for carbohydrate analysis is probably in the determination of individual sugars (Folkes & Crane, 1988). In the present study, the results obtained from HPLC were not quantified and were thus comparable only by visual comparison of sample profiles.

Ion exchange resins can be used to remove or concentrate molecules in a variety of samples,and thus allow both the concentration of dilute samples and the isolation of compounds of interest from complex materials (Bulletin 1825 US/EG, BioRad, USA). Peptides and amino acids are amphoteric and can be concentrated using anion or cation exchange resins. In this study, carbohydrate analysis of non-host exudates after cation exchange showed a decrease in the number of compounds present. This suggests that a large number of carbohydrates were trapped on the column. No real effects were observed with the root extracts. This highlights the possibility that it is influences on root tissue protein levels that are important in the AMF symbiosis (see below).

In contrast to carbohydrates, there has been recent and considerable interest in the protein profiles of mycorrhizal and non-mycorrhizal roots. In particular, endomycorrhizal colonisations have been analysed for the presence of protein molecules known to be associated with plant defence reactions in order to elucidate whether plants show similar responses to the development of the mycorrhizal symbiosis (Gianinazzi-Pearson & Gianinazzi, 1995). In the present work, the amino acid profiles of host and non-host exudates were similar. This similarity suggests that

exudation of amino acids alone is not of major importance in determining the growth responses of AMF hyphae. Extracts contained a greater number of compounds in greater quantities (Section 3.7.4.1). Surprisingly, since it has been suggested that new proteins occur in mycorrhizal roots which are not present in non-mycorrhizal roots, few differences were apparent between amino acid profiles of host and colonised host root extracts. It is possible that the inability to detect differences in amino acid profiles using the chemical techniques employed in the present study was due to the presence of novel proteins at levels below the detection limits for the methods used, rather than to the absence of specific compounds. Even using more specialised techniques, the study of changes in protein patterns which result from AMF colonisation of host plants is difficult due to the low levels of expression, and the inability to culture the symbionts in the absence of the host plant. It is also possible that at least some of the proteins are produced transiently (Dumas-Gaudot *et al.*, 1994). These novel proteins, which may be of plant or fungal origin and which are specifically linked to the symbiosis, have been termed endomycorrhizins. Evidence suggests that they arise from the *de novo* expression of some genes and the down regulation of others.

Dumas *et al.* (1989) compared the profiles of soluble proteins in non-mycorrhizal roots and those colonised by different AMF and observed that the mycorrhizal roots contained more protein than the non-mycorrhizal roots. Analysis of protein bands from tobacco and onion roots indicated that some of the new proteins in colonised roots were of fungal origin, while other novel proteins were probably of host origin (Dumas *et al.*, 1989). Changes in protein patterns during mycorrhizal infection which indicate alterations in fungal and/or plant gene expression have also been reported in soyabean and pea (Wyss *et al.*, 1990; Schellenbaum *et al.*, 1992). Soluble protein profiles of onion plants colonised by different *Glomus* species consistently contained the new host-specific proteins in the mycorrhizal tissues. Additional proteins were also detected, some of which differed with the species of *Glomus*, indicating the occurrence of fungal specificity (Dumas *et al.*, 1989). However, the quantities of proteins present were beyond the limits of detection by either ELISA or immuno-blotting techniques and their elicitation, unlike that in interactions between fungal pathogens and host plants, was at single cell level corresponding specifically with mycorrhizal colonisation.

More recent work using *Glomus mosseae* and *G. intraradices* infected roots has provided evidence that colonisation influences polypeptide/protein synthesis in a number of ways. Some existing polypeptides remain unaffected, others are enhanced or disappear, and some new ones are induced . It is possible that some of the novel proteins are indicative of a general stress reaction, and similarities with pathogenesis-

related proteins have been indicated. Although some of these induced polypeptides are likely to be more specifically related to the establishment of the mycorrhizal symbiosis (Dumas-Gaudot, 1994), nothing is known about their function or their precise role in plant-fungal interactions (Schellenbaum *et al.*, 1992).

Because of the nature of the symbiosis and the rhizosphere as a whole, biochemical implications in the plant-AMF relationship are both complex and far-reaching. The phytohormones, which are protein compounds, are chemical substances capable of co-ordinating plant growth and morphogenesis, and their discovery raised the question of their possible involvement as signals of secondary messengers in the establishment and function of mycorrhizal associations (Beyrle, 1995). The nature of any regulatory role is unlikely to be easily elucidated due to the lack of knowledge of how these substances actually work, and their ubiquitous occurrence within plants and the rhizosphere at large makes it difficult to ascertain which of the hormones present originate from one partner in the symbiosis and influence the other, and which are produced in response to colonisation. It is implicit in the definition of phytohormones that they are capable of producing a biological response in minute concentrations, therefore precise elucidation of their potential role in the symbiosis would require highly specialised and sensitive techniques.

Different approaches have clearly shown that plant or fungal protein synthesis or expression is modified during the establishment and development of the symbiosis. The most interesting modifications involve specific, localised molecules such as enzymes at the host-fungal interface. Molecular biological techniques are likely to increase understanding of the changes that occur in protein profiles through identification of gene regulation and explanation of mechanisms at the molecular level which are responsible for mycorrhizae formation and function (Gianinazzi-Pearson & Gianinazzi, 1995). Such information is likely to define more appropriately the functional status of the symbiosis, an attribute which is too often overlooked in favour of structural studies.

Mycelial studies have revealed a broad-spectrum of physiological properties and specific adaptations to defined environmental circumstances (Read, 1992). The preceding discussion serves to highlight the complexity of the biochemistry involved in these processes, and the specialised techniques required in identification of compounds and interpretation of results. Despite recent advances, biochemical aspects of the AMF-root interaction remain relatively little understood, and while the chemical techniques used in the present study were carried out in an attempt to explain some of the observed growth effects, detailed interpretation is not possible. Efforts to describe and explain the biochemistry of the plant-AMF relationship at the

level attempted in this study served to raise more questions than they did answers. The additional use of bioassays to assess growth effects on AMF of specific molecular fractions aimed to identify the basic grouping of compounds that may be involved.

Bioassays of molecular fractions of the exudates and extracts (Section 3.7.3) indicated that the >10K fraction was largely biologically inactive. This fraction is likely to have contained oligosaccharides, peptides and nucleic acid fragments.

In contrast, the <3 and 3-10K fractions had significant bioactivities. In the <3K fraction, all significant growth effects which were observed in the presence of plant root extracts were inhibitory, particularly those mediated by host root extracts. The exudates had no significant effects, again showing differences between extract and exudate profiles. This fraction is likely to have contained a wide variety of compounds, including amino acids, small peptides, monosaccharides and small oligosaccharides, nucleotides, phenolics, organic acids, vitamins and enzyme cofactors, cytokinins and other plant growth factors, alkaloids and posssibly phosphate.

The greatest degree of bioactivity occurred within the 3-10K fraction. Observed biological effects occurred predominantly with the non-host, although these effects were contradictory, with growth depression by the exudate, and promotion by the extract. Extracts were again more active than exudates. No other effects were evident. This fraction is likely to have contained oligosaccharides, peptides and phenolic conjugates. This result indicates that the observed growth depression of whole non-host exudates described in section 3.7.2.1 may arise from compounds present in the 3-10K fraction. The presence of phenolic conjugates in this fraction may be relevant, as flavonoids are phenol derivatives and were also found to be inhibitory to hyphal growth (Section 3.7.2.1). Recent work has indicated that molecules which elicit recognition responses in AMF are either small molecules with a maximum molecular weight of 500 Daltons, or do not consist of chemical diffusates. This does not exclude the involvement of flavonoids as signal molecules in the AMF-plant symbiosis, as flavonoid molecular weight is generally less than 500 Daltons (Giovannetti *et al.*, 1996).

Within-compound statistical analysis of bioassay data showed that there were significant effects on AMF hyphal growth between the different fractions of the control, indicating intrinsic problems with the use of Sorbarods as plant growth supports and for the subsequent collection of exudates. The assumption that Sorbarods would provide an inert growth medium appears to have been unfounded.

Comparing the data in this way showed that with both host and non-host exudates, molecular fractionation had a significant effect on the growth of AMF. In

both cases, the 3-10K fraction appeared to be detrimental to hyphal growth. Possible reasons for this observed growth depression are discussed above. Interestingly, the >10K fraction of the colonised host root extract appeared to stimulate growth of AMF. In the previous analysis, this fraction was the least biologically active of the three, and the colonised host extract did not significantly influence growth. The bioactivity of the non-colonised host and non-host extracts did not vary between fractions. As the non-host extract had previously been observed to increase growth relative to the control, this suggests that bioactive substances occur throughout the molecular range. Apparent contradictions observed between data analysed within and between compounds may indicate that fraction effects are masked by stronger compound effects, and *vice versa*.

Hyphal Branching and Fractal Analysis

As has been described for root architecture, defined as the explicit spatial configuration of a root system (Nielson *et al.*, 1997), it seems obvious that the spatial deployment of extra-radical hyphae of AMF will be important, given the scarcity and non-uniform distribution of the resources in the soil environment. Hyphal architecture is equally little understood because of similar difficulties involved in observation, quantification and interpretation of a complex and dynamic growth form in a heterogeneous and opaque medium (Nielson *et al.*, 1997). Although the propensity of fungi to branch has some genetic basis, which will put constraints on any postulated mechanisms for adaptation of branching patterns according to environment (Ritz & Crawford, 1990), fractal geometry may offer an improved technique for quantifying and encoding mycelial complexity and yielding ecological and physiological insights into the functional relevance of specific architectural patterns (Nielsen *et al.*, 1997). Although fungal mycelia are not likely to be true fractals, but rather approximate fractals over a finite range of scales, the use of FD as a measurement parameter will provide additional scope for the description of fungal growth patterns, potentially giving a powerful insight into quantitative aspects of the space filling properties of fungal hyphae.

There have been relatively few attempts to examine the fractal geometry of either root systems (Tatsumi *et al.*, 1989; Fitter & Strickland, 1992; Nielsen *et al.*, 1997) or fungal mycelia (Ritz & Crawford, 1990, Crawford *et al.*, 1993; Hitchcock *et al.*, in press), as a result of which there is little reference material against which to interpret results and relate them to natural conditions. In 1992, Fitter & Strickland reported on the estimation of root system FD *in situ* in the soil, thus retaining the natural geometry of the root system, and demonstrated the applicability of fractals to

studies of root systems measured in realistic conditions. They concluded that fractal analysis may have a valuable application in root system studies, providing an index representative of the complexity of the branching system, and able to integrate topology and geometric parameters. Despite the lack of recent publications dealing with FD as a measurement of growth characteristics in filamentous microorganisms, there is also some evidence that FD can provide an additional and satisfactory parameter for the characterisation of mycelial trees during specific phases of growth (Reichl *et al.*, 1990). Using *Streptomyces tendae* Tü 901, Reichl *et al.* (1990) found that, on average, hyphae always used the same proportion of their area for nutrient supply. The dependency of the FD on culture conditions was not examined, but was recognised as an area for future work. Ritz & Crawford (1990) used fractal analysis to define the relationship between fungi and substrate with *Trichoderma viride*, and concluded that the fractal growth form optimised nutrient capture by optimisation of the balance between exploratory and exploitative growth.

In this study, visually observed differences in growth form of AMF mycelium were tested using the program "Fungus", developed specifically in conjunction with this work (Delas *et al.*, 1996). The results showed that the presence of host root exudates appeared to cause a reduction in hyphal branching, even though this was not significant, that lent credibility to the hypothesis that AMF hyphae are able to adapt their growth form in response to environmental signals. Giovannetti *et al.* (1993b) showed that host signals trigger differential branching patterns in the development of AMF hyphae from spores prior to the formation of infection structures. With extra-radical hyphae, a lesser degree of branching may be indicative of a change from an absorptive type hyphae to an infective type hyphae, as described by Friese & Allen (1991), enabling the initiation of new infections in the proximity of a host plant. Significant differences were observed in the fractal dimension (FD) of the AMF mycelium in the presence of host exudates compared with the control. Host exudates decreased the FD to a value closer to 1, indicating a greater linearity of growth form, and again implicating host-derived factors in changes in hyphal growth form commensurate with a structure-function relationship.

Contradictory results were evident between experiments. The follow up experiment which tested the effects of host and non-host exudates and the flavonoids hesperetin and naringenin on hyphal growth did not show any significant differences in either branching or FD. Trends indicated that host exudates and hesperetin increased branching, and non-host exudates decreased branching. This contrasts with the trend towards decreased branching in the presence of host exudates observed in the previous experiment. Non-host exudates were previously observed to decrease hyphal

length. That this may be due to decreased branching raises the possibility of a more complex hyphal response to the presence of the non-host. As previously discussed, this change in growth pattern may be triggered by non-host recognition, resulting in minimum use of resources to achieve maximum hyphal length in the search for a host plant, stimulated by the presence of the non-host.

It is possible that these apparent but non-significant trends are irrelevant, and that none of the compounds tested were in fact elicitors of any biological response affecting either the degree of branching of the AMF hyphae or their distribution within the mycelium. However, given the growth effects which are known to occur in germination of AMF from spores, it is perhaps more likely that methodological and variability problems were masking growth effects in these experiments. Some of these problems have been discussed previously, and others concerning the program itself are dealt with below.

More pronounced effects were evident when root extracts were tested. Both the host and non-host extracts significantly increased FD. Thus, compounds present in the extracts were altering the form of the AMF mycelium, apparently increasing its space filling abilities by pushing it towards a more uniform "surface" oriented distribution. This may occur because in the biochemical array presented by a root extract, the AMF recognises an unlimited stimulus which negates the need for exploration and favours exploitative growth, enabling the fungus to continue to produce higher orders of branching until d=2 (ie. equivalent to a "surface" distribution). In contrast the colonised host root extract did not significantly influence FD, again indicating the possibility of biochemical modification as a result of colonisation by AMF. No significant effects on either hyphal length or branching were observed in the presence of the host extract, but there was an apparent trend towards a decrease in branching which matched apparent effects of the exudate. The non-host extract increased both hyphal branching and FD, indicating differences in response of AMF between extracts as well as exudates. The increased hyphal branching elicited by the colonised host root extract again shows differences as a result of the colonisation process.

In their studies of root systems, Fitter & Strickland (1992) found evidence that increases in FD reflect increases in branching complexity. However, because of the relatively recent use of the fractal concept in analysis of biological growth forms, the interpretation of fractal values is difficult, and understanding the relationship between computed values of FD and the functional attributes of the system under examination remains a challenge (Nielsen *et al.*, 1997).

The "Fungus" program was specifically developed in conjunction with this project and enabled the extraction of additional useful information from images of AMF mycelia which were initially generated for more general measurements of total hyphal length using existing facilities within the department. By the admission of the program's writer, it could be improved in many ways, particularly by orienting it more specifically to the study of fungal mycelia rather than to the purely mathematical interpretation of the images obtained. The program placed emphasis on breaking the structure of the mycelium down into its component linear segments, which were not necessarily appropriate biological measures of the mycelial structure. For example, a single hypha with two branches, in reality composed of a main hypha with two junctions to branch hyphae, would be interpreted by the program as five hyphae by literal allocation of the term hypha to segments of the hyphae both between nodes and between nodes and hyphal tips. Thus in this simple example, the program would count five hyphae, of which four would be termed branches, and one an "inner hypha" (Figure 3.11a & b). To achieve a more biological orientation to the program would require further liaison between the mathematicians and the mycorrhizologists in terms of the biological interpretation of the networks and the context within which the data are created. A more powerful programming language run behind a Visual Basic interface would also enable the introduction of more elaborate algorithms.

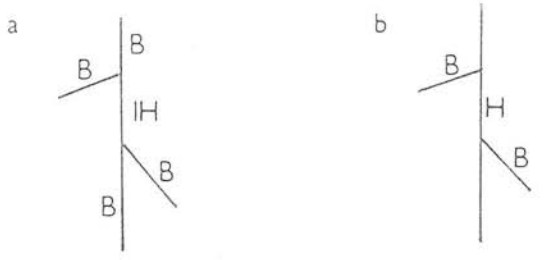

Figure 3.11: Simplified diagrammatic representation of definitions of hyphae (H), inner hyphae (IH) and branches (B) used (a) in the Fungus program and (b) in biological interpretation of the network

One of the problems encountered concerned the generation of hyphal branching data. In this study, the number of branches per mm of total hyphal length was used as the parameter for comparison. This was calculated using the values of

total hyphal length and number of branches computed by the program. More accurate comparative data may have been achieved if it had been possible to separate "total" and "non-branch" length, and thus calculate the number of branches per mm of non-branch hyphal length. It seems likely that the number of branches occurring in a given treatment will in itself have influenced the total hyphal length, in turn biasing the total length to which the branches were allocated. However, separation of total and non-branch hyphal length was beyond the capacity of the program because of its inability to identify any "main stem".

The program worked by allocation of different classifications to the components of the mycelium. Firstly, the lengths of all parts of the network were summed to give "total length". Then, lengths of any hyphae which were connected to the nearest part of the network at one end but "free" at the other were summed, giving "total length of branches". Thus, total branch length in fact describes only the length of all parts of the hyphae that start at an internode and end at a tip. The length of any part of a branch between the node from which it subtended and any node with a higher order branch along its length would therefore be discounted in the branch length calculations (Figure 3.12). In addition, the tip of any hypha would automatically be classed as a branch because it would be unconnected at one end, but continuous with the network at the other.

Using the number of branches per mm of total length would be acceptable as a measure assuming equal branching rate for increasing orders of branching ie. primary, secondary, tertiary etc. There is no evidence to support such an assumption. The inability of the program to recognise a "main" hypha as compared with its branches must therefore introduce error into its calculations. In studies of streptomycetes, the longest connected path through the object has been considered as the main hypha (Packer & Thomas, 1990). In this case it was argued that despite the questionability of using this parameter as the main hyphal length, it probably provided the best measure of "effective" length (Packer & Thomas, 1990). The application of such a definition in the present work would be complicated by the complexity inherent in the AMF mycelium, which would make identification of a main stem using this parameter unlikely to be an accurate representation of the true morphology. The assumption that there would be a single main stem would also be inaccurate in this case, as a feature of hyphal growth from root pieces is the growth of multiple hyphae from a number of exit points. One potential method of overcoming the fact that the program is not "aware" of differences between main hyphae and their branches, and so to make relevant calculations of the length of the non-branch hyphae, would be to develop a program able to "unfold" the hyphae into 3D form, simply from a 2D black and white

image. However, this would be a complex programming task which is yet to be attempted (Delas, pers. comm.).

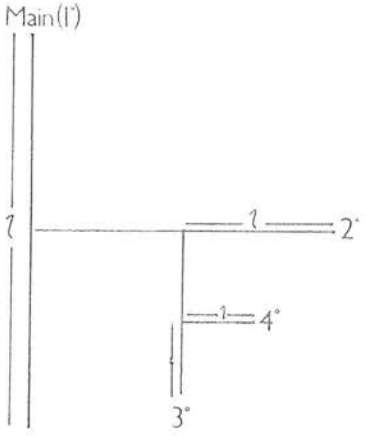

Figure 3.12: Simplified diagrammatic representation of allocation of hyphal length (l) to branches in the Fungus program

Calculation of FD would obviously give a greater insight into growth patterns if it could be carried out in 3D. It has been recently suggested that in order to compute FD in 3D space, it is necessary only to compute the FD of one projection onto a plane. The possibility that FD in different spatial dimensions is related in a predictable way has important repercussions on measurement and analysis of biological growth forms because of the implication that 2D estimates of FD can be used in 3 dimensional estimations of complexity (Nielsen *et al.*, 1997). Recent work suggests that, in theory, FD in 3 dimensions can in fact be estimated from 2D measurements. However, there are practical difficulties involved in doing so (Nielsen *et al.*, 1997).

A possible drawback to the use of the program in calculation of FD was that as the mycelial maps were so large, part-images were selected for analysis which were representative of the hyphal mass as a whole. Values were averaged across different replicates, using unweighted means. Although each selected section of the hyphal map was calibrated to the magnification at which it was imaged, averaging in this way may have brought inaccuracies into the results where images were of different sizes. The choice of radii controlled the scale at which the hyphae were viewed in calculation of FD, which looks at how the density of the hyphae changes in moving from the smallest to the largest radius. The largest radius (x) should be such that the amount of hyphal structure within the square "box" of radius x is sufficient to give an impression of the complexity of the whole network. The box should not need to cover the whole image. Had this been the case, it would have both considerably slowed the program

down, and allowed areas as yet uncolonised by the hyphae to have too much influence in the calculations. Even with a maximum box size much smaller than the actual image size the whole image will still be assessed, as the program works by looking at the increase in hyphal length with box size about every hyphal pixel in the image. The choice of radii used in this study, ranging from 5 to 55 in 5 stages, was recommended by the program's author. However, image size may again have impacted on results, and it is possible that more work on adjustment of radii may have been appropriate. Hitchcock *et al.* (in press) suggest that the optimum radius depends on the scale at which the mycelium displays a range of behaviour.

A second potential problem arising from the use of part images was that this generated additional endpoints through the formation of "cut-off" hyphae. Because the program calculated rather than counted the "real" number of branches, using branch points (B), endpoints (E) and segments (S) in the equation E+2B-S-1, this may have biased the results by incorporating additional endpoints into the equation which were in fact artefacts of the use of part images, thus resulting in inaccurate calculation of the number of branches. It was unclear how this may have affected results from the program. Assuming that all cut-off hyphae generated by the use of part images ultimately have an endpoint outside the image anyway, whether the endpoint is within or on the boundary of the image may be irrelevant. Packer & Thomas (1990) used an active measuring frame within the image frame to overcome this problem by ensuring that microorganisms were not truncated by the edge of the image. To avoid bias, the distance between the measuring frame and the edge of the image was greater than the size of the largest organism. In this study, the generation of "new"endpoints at the edge of the artificially created part image may only have been relevant in branching calculations if the same hyphae appeared again in another image and was thus counted twice. Because adjacent images were not used here, the problem may have been more a theoretical than a practical one.

Exploration of this as a potential problem identified a further possible drawback of the program. This was centred on the program's assumption that the image would be composed of a single connected component, and its consequent inability to deal with the existence of multiple hyphae within the image. While the user can recognise that the components are connected outwith the part image that is being analysed, the computer (and thus the formula) cannot "see" outside the part image and is thus unaware of this connection. Provided that the user knows how many components (ie. hyphae) there are in the image then the formula, which is based on one hypha, can be adjusted. For n hyphae, this adjustment will be n-1. In the present work, and as previously described (Section 3.5.5.2), the number of branches

calculated by the program had to be adjusted by this correction factor in order to obtain the correct result.

A number of problems were thus recognised in the use of the program. Had it been possible within the timescale of the study period in which the program was developed, these would have been discussed with its writer and a more appropriate version would have been evolved. However, this was not the case. Despite the problems, the program did enable generation of additional data from existing images, and it is hoped that the recognition and description of these alongside the results will make it clear that the author appreciated these shortcomings.

Chapter 4

4.1 Overview

Individual plants, animals and microorganisms are often considered as separate entities. More commonly, however, organisms live in association with other species, often in highly successful and specialised symbiotic relationships that can completely alter their life strategies. Symbioses are often key factors in the structure and functioning of ecosystems (Sanders *et al.*, 1995). The importance of symbiotic interactions in the functioning of natural ecosystems and the maintenance of biodiversity is highly relevant to other disciplines involved in environmental management. In terms of AMF, this relates to the development of effective practices for sustainable agriculture, through increasing the understanding of a range of mycorrhizal relationships and their interactions with individual plant species and plant community dynamics (Smith, 1995). Results of these studies will ultimately be of practical significance in economic plant production systems. This is discussed in greater depth in Section 4.2.

Mycorrhizal colonisation is a multi-step process during which many different signals cause a cascade of recognition events between the host and symbiont. The mycorrhizal state is therefore the product of a number of signals of differing nature, acting at various stages of this interaction and eventually leading to the development of the functional symbiosis (Giovannetti *et al.*, 1993a). Developing a more detailed and potentially practical understanding of AMF requires study at two levels, that of the ecosystems at which AM fungal populations interact with other members of the community, and at a more detailed level, unpicking the systems to understand their more fundamental aspects (Smith, 1995). Currently, we are able to make broad generalisations concerning the organisms, their symbiotic associations, structure and function, but mycorrhizal research presents a continuous challenge, in which questions are raised and techniques must be developed to provide answers. Maintaining links with other study areas that while not interested in mycorrhizae *per se* require an integration of knowledge, is an important aspect of this research (Smith, 1995).

Mycorrhizal associations are amongst the most frequently described symbioses. The productivity and diversity of natural ecosystems frequently depends on their presence and activity, reason enough to justify continuing mycorrhizal research (Smith, 1995). There are indications that AMF play a major role in the composition of plant communities and that they subsequently affect the various processes critical to forming a stable ecosystem (Allen *et al.*, 1992). Mycelial studies reveal an increasingly broad spectrum of physiological properties and specific adaptations to defined environmental circumstances, but the mechanisms by which

these fungal interactions are expressed remain little understood (Allen *et al.*, 1989) and basic questions still need to be answered. To increase our understanding of the major terrestrial ecosystems depends on a greater awareness of the activities of these fungal mutualists (Read, 1992). AMF play a major role in the composition of plant communities, and this has knock-on effects on various ecosystem processes that are critical to the formation of a stable ecosystem. Ecosystem processes are highly dependent on the community composition, which in turn depends on the presence of AMF and plant growth responses to it (Allen *et al.*, 1992). An important factor in plant growth response is its dependence on how specific plant tissues respond to individual fungal invasions, responses dictated by molecular interactions between invading hyphae and molecular recognition features determining the compatibility of the interaction. This is indicative of the relationship between processes occurring at the ecosystem level and those at the molecular level at the interface between two different and unrelated organisms (Allen *et al.*, 1992).

It has been suggested that an explicit recognition of the differing scales at which interactions occur between AMF and their plant partners is the basis of the research challenge facing mycorrhizologists in their attempts to understand how mycorrhizas affect ecosystems (Allen *et al.*, 1992). Only understanding AMF and their relationships with other components of ecosystems at a wide range of scales will lead to the development of more appropriate land management strategies that meet both human and environmental needs (Allen *et al.*, 1992). If one aspect of the mycorrhizal symbiosis is abundantly clear, it is that the AMF-plant system does not operate in isolation, and that each step of the way relationships with other members of the ecosystem are influencing both partners of the symbiosis and their interaction with each other. Explicit recognition of the different scales of interaction between AMF and plants will determine the research questions required to increase our understanding of how mycorrhizae affect the processes important to both natural and man-made ecosystems (Allen *et al.*, 1992).

4.2 "Real World" Applications: The Relevance of Mycorrhizal Research to Sustainable Agriculture

It is the author's belief that aspects of research should be applicable at a wider level than that dictated by the confines of the laboratory. This chapter aims to describe the role of AMF within the context of agricultural systems, and to link this with

aspects of mycorrhizal research. The relevance of the present study on the AM fungal mycelium will be discussed in this context.

As a result of technological advance there has been a considerable increase in world agricultural production over the past few decades. This has been achieved through improvements in productivity and by the development of farming techniques that bring previously marginal land into production (Lovato *et al.*, 1995). Modern agricultural practices in developed countries have aimed at the production of increased yields per unit of land, but to maintain artificially high levels of production requires the use of increasing inputs of chemicals and energy, resulting in over-production and an increasing cost of agricultural support (Parsons, 1985). Yield improvements obtained at the expense of energy efficiency (ie. a decrease in the ratio of total energy input to yield), cannot be maintained (Mosse, 1986).

An increased reliance on agrochemicals over the past half century has allowed agricultural practitioners in developed countries to ignore all but the most superficial of ecological relationships in most cropping systems. The use of mineral fertilisers has negated the need to understand soil or organic matter dynamics or changes in soil microbial communities that are associated with agricultural practices (Paul & Robertson, 1989). Although natural ecosystems are ill-defined and ever-changing, the patterns and processes that are still discernible remain the most appropriate standard in the development of sustainable agricultural systems (Jackson & Piper, 1989).The term sustainability defines the successful management of resources to satisfy changing human needs, while maintaining or enhancing the quality of the environment. Increasing environmental degradation and instability resulting from anthropogenic activities, and especially increasing fragility of the soil ecosystem, has led to an increased awareness of the need to develop practices resulting in more sustainable natural and agricultural ecosystems (Gianinazzi & Schüepp, 1994).

To try and improve agricultural systems by understanding them on their own terms, rather than by acknowledging their ultimate reliance on the natural systems from which they arise, will not provide the answer. For many and varied reasons there is increasing interest in bringing together the more basic and applied aspects of the environmental sciences, and this is particularly true for ecology and agriculture, between which there has traditionally been limited communication (Coleman, 1989). Meeting the twin challenges of economic and environmental sustainability requires that agronomists consider the interactions of all the important biological and physical components of their cropping systems, and integrate this at the community level. The application of ecological theory to agronomic systems is central to this integration (Paul & Robertson, 1989).

The intensification of conventional agricultural systems has had a major impact on soil management, resulting in a general shift away from soil fertility maintenance towards crop nutrition that has degraded soils from their balanced pre-cultivation state (Arden Clarke, 1988; Bethlenfalvay & Linderman, 1992). On a world scale, there is now a need to develop sustainable agricultural systems with lower (fertiliser) inputs (Sanders & Sheikh, 1983). Currently practiced intensive systems are simple in ecological terms. Whether or not these systems can be modified to meet the goals of sustainable agriculture is in question (Atkinson & Hooker, 1993) as alternative methods, eg. the manipulation of microorganisms, cannot be used as direct substitutes for agrochemicals but must rather be integrated into the whole system (Atkinson & Hooker, 1993). The concept of systems design is of primary importance in the development of low input systems. These systems have the potential to decrease environmental disruption by placing reliance on a greater understanding of the underlying biological processes and cycles on which they ultimately depend. External inputs are then required only to supplement the management of internal features (Lampkin, 1990). Sustainable agriculture aims to manage the land as an ecological system, and to decrease inputs without a significant decrease in productivity. How to achieve this long term without environmental degradation presents a challenge that requires the development and perfection of biologically-based farming systems that can combine productivity and profitability with sustainability (Youngberg, 1990).

The soil is the resource basic to agricultural systems, and as it is non-renewable the maintenance of its fertility is crucial to sustainability. In purely agricultural terms, the fertility of a soil can be defined as its capacity to produce desired crops (Russel, 1973). Such a rudimentary definition, however, simplifies the complex nature of the plant-soil system. In conventional systems, the soil is regarded as an inert matrix for accommodating crop root systems and for the retention of a proportion of applied nutrients prior to root uptake (Arden Clarke, 1988). Cultural practices implemented in agricultural systems are thus aimed at production rather than protection and as a result are detrimental to mycorrhizal formation and propagule survival (Bethlenfalvay, 1992). Organic inputs that are characteristic of sustainable systems are chemically more complex than synthetic agrochemicals, thus placing increasing importance on the role of the root system and its associated microorganisms. It is likely that the rapid increase in the concentration of plant nutrients associated with synthetic fertilisers is responsible for a reduction in mycorrhizal development. The slow release of nutrients that characterises organic fertilisers is unlikely to have this effect. Plants given farmyard manure have been observed to form mycorrhizal associations with more arbuscles than those given

equivalent amounts of mineral fertiliser, and to form more connections between the internal and external mycorrhizal mycelium (Mosse, 1959). The mycorrhizae may benefit nutrient uptake from other slowly available sources in the same way that they increase uptake from rock phosphate (Mosse, 1986). It is likely that AMF promote the uptake of any nutrients that move to plant roots primarily through diffusion, and they are thus able to increase both nutrient uptake and utilisation (Abbot & Robson, 1986; Sanders & Sheikh, 1983).

Additional benefits in terms of the role played by AMF in enhancing nitrogen fixation by nodule forming bacteria are also recognised, as a synergistic effect results in a tripartite relationship that is more effective than the combination of any two of the components (Linderman, 1992). The plant-*Rhizobium* system benefits as a result of correction of nutritive deficiencies within the system, and thus almost all nodulated legumes grow better if they are also mycorrhizal (Linderman, 1992). Mycorrhizal effects include increases in growth and yield, foliar nitrogen concentration, and nodule size and number; efficiency of the enzymes involved in nitrogen fixation is also increased as a result of the increase in phosphorus supply which stimulates the nitrogen-fixing process (Rhodes, 1980).

Microbial ecology provides an example of a field that spans both agronomy and ecology (Paul & Robertson, 1989). No natural vegetation systems, and few man-made ones, are without a large and varied microbial population incorporated within a complex system of plant and substrate (Tinker, 1984). Increases in soil fertility are thought to be largely comensurate with increased microbial biomass and activity, and soil microbes and fauna play a critical role in buffering the soil system against natural and cultural stresses (Bethlenfalvay & Linderman, 1992). Manipulation of roots and rhizosphere organisms may help sustain currently achievable production levels when inputs are decreased. Of the multitude of organisms in the agricultural ecosystem, AMF stand out because of their ability to bridge plant and soil to the benefit of both (Bethlenfalvay & Schüepp, 1994). Mycorrhizal infection induces changes in root morphology, resulting in a greater degree of branching that enhances the ability of the plant to exploit the soil resource. Changes in branching characteristics also affect root system plasticity by promoting the development of short-lived higher order roots. Decreased root longevity is thought to increase resistance to soil borne diseases.

Although modifying rhizosphere organisms for the conservation of soil nutrients has potential in low input systems (Atkinson & Hooker, 1993), many of these soil bacteria and fungi immobilise mineral nutrients as they consume carbon and may thus compete with plants for macro-nutrients where these are limiting. In contrast, mycorrhizal fungi obtain carbon directly from the plant photosynthates and

can therefore efficiently couple soil mineralisation and nutrient uptake by plant roots (Reid, 1990). In facilitating this plant-soil link, the AMF provide the tools and technology for use in crop production and soil conservation and thus have a valuable role in sustainable agriculture (Bethlenfalvay, 1992; Bethlenfalvay & Linderman, 1992).

The use of soil organisms such as AMF in reducing current reliance on nutrient imports has great potential in the establishment of a more sustainable agriculture. The benefits of mycorrhizal colonisation are generally determined by growth response or nutrient uptake, representing a host rather than a rhizosphere orientated view (Miller & Jastrow, 1992). In fact, external mycorrhizal hyphae offer the additional advantage of aggregate stabilisation which can persist for several months after host plants have died (Tisdall & Oades, 1980), allowing the ultimate possibility of matching AMF to agricultural systems in the interests of soil fertility and sustainability. The extra-radical phase is an important contributor to the process of creating a stable soil aggregate structure (Tisdall & Oades, 1982; Miller & Jastrow, 1990) and both roots and external hyphae play a primary role in the mechanism of soil aggregate development through physical entanglement of particles (Miller & Jastrow, 1992). This process of packing soils into aggregated units is identified as a method of accumulation of organic matter, as residues are protected from microbial activity (Oades, 1984). Management is thus important in maintaining soil organic matter content, and ultimately in AMF colonisation and fertility (Tisdall, 1991).

The specific placement of hyphae in the rhizosphere is just as important as their abundance (Abbot & Robson, 1985; Jakobsen *et al.*, 1992). If hyphae are able to respond to environmental influences, then this ability may exemplify the mechanism underlying the creation of the hyphal network. Microsites of organic debris may thus provide the necessary step in the proliferation of external hyphae and in the process of physical entanglement and enmeshment of soil particles and aggregates (Miller & Jastrow, 1992). Persistence of a mycelial network between seasons may also be an important factor in the early phosphorus nutrition of annual crop species. Infected seedlings will immediately become linked to an extensive network of mycorrhizal hyphae which are able to pass across the depletion zone. This forms around roots as result of more rapid rate of uptake at the root surface than flow rate through the soil to the root (Mosse, 1986). Such hyphal networks are more important, in terms of their rapidity of effect, than are isolated propagules.

The relevance to agriculture of mycorrhizal effects depends largely on the degree of disturbance within the system, as this will decrease the benefits to sustainability (Evans & Miller, 1990). The abundance of extramatrical hyphae tends to

follow a trend opposite to that of the soil fertility gradient (McNaughton & Oesterheld, 1990), and mycorrhizae could thus have a particular role in less intensive systems where phosphorus is more likely to be limiting, and where their significance in phosphorus nutrition is increased (Evans & Miller, 1990). Phosphorus contained within the hyphal system is protected against further immobilisation in the soil, and the presence of mycorrhizae may therefore promote more efficient use of applied fertilisers (Mosse, 1986). The positive association between length of extramatrical hyphae and ratio of plant:soil nutrient content implies that mycorrhizae functionally compensate for widescale variations in soil fertility, so that nutrient status of plants is not proportionally affected by differences in the soil nutrient status. It has been postulated that mycorrhizae may thus act as stabilisers of nutrient cycling through variations in the density of their extramatrical hyphae across edaphic gradients (McNaughton & Oesterheld, 1990).

Potential benefits to plant growth and yield through fieldscale inoculations of AMF depend to a large extent on field conditions, competitive infection by indigenous species and on the level of available nutrients, particularly phosphorus. An increasing concentration of soil phosphorus may decrease mycorrhizal infection to levels unable to enhance uptake of other limiting nutrients (Abbot & Robson, 1986). To obtain the last 5-10% of production from plants which are very low in mycorrhizal inoculum will require a very high fertiliser input. Conversely, using a mycorrhizal system to its fullest extent it is possible to achieve 90% of maximum production with only a 50% fertiliser input. In most agricultural systems mycorrhizal infection is likely to be reduced by the addition of excess nitrogen, thus initiating a cycle in which additional nitrogen decreases the crop mycorrhizal status and requires added phosphorus to make the applied nitrogen effective (Mosse, 1986). Micro-nutrients may also become limiting due to decreased efficiency of the depleted mycorrhizal system (Rhodes, 1980). The use of NPK fertilisers is thus implicated in disruption of naturally-mediated nutrient uptake systems, with the result that increased reliance is ultimately placed on such synthetic nutrient imports. In addition, where phosphorus is no longer a limiting nutrient those AMF which do colonise plant root systems become increasingly parasitic and act as a drain on the plant photosynthetic system. The ability of hyphae to exploit inherent soil reserves, in conjunction with the degree to which they can remain functional in nutrient uptake and transport under extremes of soil environmental conditions, is therefore important in agricultural terms.

Over the past two decades there have been major advances in the biological sciences. These have generated a new set of tools for understanding, utilising and manipulating biological resources - biotechnology. This has allowed the integration of

the applied biological sciences into a framework that draws on an improved understanding of fundamental biological processes (Wood & Cummings, 1992). Biotechnology similarly provides an interface between the basic and applied mycorrhizal sciences. AMF are fundamentally difficult organisms to work with as their obligate biotrophic status and as yet little understood genetic systems are significant obstacles in both basic and applied research (Wood & Cummings, 1992). Advances in important aspects of AMF biology will build the foundations of an ability to enhance their utilisation, and thus enable the exploitation of the plant-mycorrhizal system for agricultural benefit. One of the biological tools now being integrated into biotechnology is the development of commercial inoculants of AMF for use in agriculture, horticulture, forestry and reclamation. The impetus for commercialisation of AMF inoculum has been triggered by reports in the literature which associate the AM symbiosis with positive plant growth effects.

New developments in the potential use of AMF as biological tools in the context of technology for sustainable agriculture should enable agricultural production at adequate levels while reducing chemical inputs. Their potential as biological enhancers of agricultural production, although well recognised, is not well exploited. This is largely because of currently used agri-practices and their environmental implications. Before mycorrhizal technology can be applied on a large scale, it will be necessary to identify a selection of plant and fungal species, to investigate inoculation production and application procedures, and to develop agricultural management techniques which take the requirements of the symbiosis into account (Lovato *et al.*, 1995).

The identification and determination of relative importance of AM fungal species is often derived from spore counts, which do not necessarily reflect either their relative dominance or their functional importance (Jeffries & Dodd, 1996). The extra-radical AMF hyphal network has been described as the most important of the AM fungal structures in terms of plant nutrition (Read, 1992) and as a vital component of the soil ecosystem (Barea & Jeffries, 1994). It acts within a close "cause-and-effect" interchange of mineral nutrients, carbon compounds and plant-microbe signals (Jeffries & Barea, 1994), and its behaviour is critical to the success of the symbiosis. The production of a well-developed hyphal network will occur only under conditions which favour its growth, and will be sensitive to adverse conditions and to soil disturbance. A failure of the network may result in the failure of a plant community to establish. It is thus not only important to identify plant growth effects specifically related to the development and status of an extra-radical hyphal network (Barea & Jeffries, 1995), but also to ascertain the environmental factors that are likely to

influence the development of the extra-radical mycelium itself. This is an area of mycorrhizal research that has been little exploited, but which is critical to further understanding the actual role of AMF in the field.

4.3 In Conclusion....

A major factor highlighted by the present work was the fundamental difficulty associated with studies of specific aspects of AM fungi. The complex relationship between plants and the extra-radical phase of their AM fungal symbionts that has become apparent from this study must have a basis in reality, but may also be affected by the artificial environment in which it was carried out. It is, however, hoped that the work will contribute to our understanding both of the mycorrhizal symbiosis as a whole, and of the interactions of individuals and species.

The present study primarily highlighted the differential responses to environmental factors that occur between different phases of the AM fungal life cycle. Although recognition of the importance of signalling between the two partners of the symbiosis is by no means a new phenomenon in mycorrhizal research, this work looked at fundamental aspects of fungal-host recognition and response which have not previously been examined, and in particular highlighted differences that are associated with inoculum type. This has obvious implications in terms of whole ecosystems and the potential impact of agricultural practices on their functioning. Disruption of an established extra-radical mycelium will alter both balance and structure of the population of fungal propagules in the soil, and will thus affect the environmental responses that might be expected to occur. That the extra-radical mycelium can respond to its environment has been recognised for a number of years, but the precise nature of the response has been less clear. Further elucidation of the nature of signals and fungal responses to them will improve knowledge of dynamics at both species and community level, and indicate the precise role of AMF in plant community structure and function.

Currently there is little, and varied, evidence concerning the mechanisms of contact between the partners in the plant-AMF symbiosis. An interesting finding which arose from this work was that growth responses differed considerably with the stage of hyphal development when the test substance was applied. This occurred to the extent that the expression of a particular response was completely prevented when test substances were applied to root piece inoculum from which hyphal growth was already established, compared with their application prior to the onset of growth. If such behaviour also occurs under natural conditions, it is likely to have a profound effect on the relationships between AM fungi and members of the plant community.

Extra-radical hyphae have a diverse role in the symbiosis, with responsibility for both the initiation of infections and the uptake of nutrients. If, as appears possible from this work, their precise role is determined by their state of development, then where the mycelium is well developed it may be unresponsive to plant factors which would otherwise trigger its infective potential, responding rather to nutritional influences, and *vice versa*. Thus, where newly germinated hyphae are subjected to plant related factors they may be more responsive, changing their growth form to the infective state.

Non-host suppression of hyphal growth may be interpreted as a response to the lack of host potential, dictating a change in form that will maximise growth while minimising resource use in the search for a compatible host plant. True effects are not clearly defined by the results of the present work, but these results do nevertheless provide early indications for the existence of recognition and response between host and non-host plant factors and extra-radical hyphae of AMF. How exactly these responses are triggered, and how they may be manipulated to allow more complete integration of AMF into agricultural systems that are likely to include non-host plants in at least part of the rotation, are important questions which provide scope for continuing research.

It will be a continued challenge of mycorrhizal research to transfer the observations obtained under controlled conditions to the reality of the external environment (Bethlenfalvay & Schüepp, 1994). The response of the fungal mycelium to environmental factors is not the only subject area for which there is limited knowledge. Relative nutrient transport efficiency between species may vary, which will have repercussions on the real role of the symbiosis in low-input systems that place a greater reliance on it. Hyphal longevity is also a little understood aspect of AM fungal growth, but one which is likely to be as important as its ability to respond to environmental cues. The use of AMF as a potential tool in sustainability lies with increasing our understanding of fundamental aspects of the extra-radical mycelial structure, function and environmental responsiveness.

The effects of AMF in ecosystems are not restricted to plant growth responses, but the full range of their hidden below-ground contribution is yet to be comprehensively understood and acknowledged. AMF in sustainable ecosystems have been described as "the universal compensators needed to accomplish the mission of sustainable agriculture" (Bethlenfalvay & Lindermann, 1992). It is to be hoped that continued research efforts will allow us to fully understand and utilise them in this role.

Appendices

Appendix 1

Hoaglands Nutrient Solution

	$g\ l^{-1}$
KNO_3	0.51
$Ca\ (NO_3)_2$	0.82
$Mg\ SO_4.\ 7H_20$	0.49
$KH_2\ PO_4$	0.136
Ferric tartrate	$1ml\ l^{-1}$ 0.5% solution
Hoaglands A-Z Micronutrients	$1ml\ l^{-1}$

Hoaglands A-Z Micronutrients

	$g\ l^{-1}$
H_3BO_3	2.86
$MnCl_2.\ 4H_2O$	1.81
$ZnSO_4.\ 7H_2O$	0.22
$CuSO_4.\ 5H_2O$	0.08
$H_2MoO_4.\ 2H_2O$	0.025

Appendix 2

Programming Information for the Program "Fungus" (Delas *et al.*, 1996)

A2.1 edit.frm

A2.1.1 Formload

```
Sub Formload ( )
Dim c As Integer

picture1.ScaleMode = 3
picture1.Width = sizex * ScrX: picture1.Height = sizey * ScrY
If picture1.Width < picture2.Width + 200 * ScrX Then
frmedit.     Width = picture2.Width + 240 * ScrX
Else
frmedit.Width = picture1.Width + 40 * ScrX
End If
frmedit.Height = picture2.Height + picture1. Height + 100 * ScrY
picture1. Picture + LoadPicture (pathout (0))
tmem = 0
End Sub
```

A2.1.2 Paint

```
Sub Paint (ByVal t As Integer, ByVal col As Long)
Dim i As Integer, j As Integer
Dim a1 As Integer, b1 As Integer
Dim c As Integer, l As Integer
Dim x As Integer, y As Integer
Dim startx As Integer, starty As Integer
Dim ex As Integer

        c = 0: l = 0
        i = ends (t, 1): j = ends (t, 2)
        If branch (t) = 0 Then i = onepoint (t, 1): j = onepoint (t, 2)
        startx = i; starty=j
        Do
        ex = 0: x = 0: y = 0
        For a1 = -1 To 1
                For b1 = -1 To 1
                If ((i + a1 < > c Or j + b1 < > l) And (a1 < > 0 Or b1 < >0)
And (i + a1 < > startx Or j + b1 < > starty)) Then
```

```
                If pic (i + a1, j + b1) < > 0 Then
                        x = i + a1: y = j + b1
                                If g (x, y) = t Then
                                        ex = 1
                                        picture1.PSat (i - 1, j - 1), col
                                        c = i: l = j
                                        i = x: j = y
                                        Exit For
                                End If
                        End If
                End If
                Next b1
                If ex = 1 Then Exit For
        Next a1
        Loop While ex = 1
End Sub
```

A2.1.3 Others

```
declarations
Option Explicit

Dim tmem As Integer

**************************************************************

Sub CmdOk_Click ( )
        Unload Me
End Sub

**************************************************************

Sub CmdSave_Click ( )
picture2.Cls
picture2.Print "saving picture...please wait."
SavePict (pathout (0))
picture2.Cls
picture2.Print "saving and displaying done."
End Sub

**************************************************************

Sub Form_Load ( )
```

```
Dim c As Integer
picture1.ScaleMode = 3
picture1.Width = sizex * ScrX: picture1.Height = sizey * ScrY
If picture1.Width < picture2.Width + 200 ScrX Then
    frmedit.Width = picture2.Width + 240 * ScrX
Else
    frmedit.Width = picture1.Width + 40 * ScrX
End If
frmedit.Height = picture2.Height + picture1.Height + 100 * ScrY
picture1.Picture = LoadPicture (pathout (0))
tmem = 0
End Sub

**************************************************************

Sub Picture_1MouseDown (Button As Integer, Shift As Integer,
x As Single, y As Single)
Dim c As Integer, 1 As Integer

picture2.Cls
picture2.Print "zooming..."
zoom = 30
If zoom * 2 > sizex Then zoom = sizex \ 2
If zoom * 2 > sizey Then zoom = sizey \ 2
c = x + 1: 1 = y + 1
If c - zoom < 1 Then
  c = zoom + 1
Else
        If c + zoom > sizex Then c = sizex - zoom
End If

If 1 - zoom < 1 Then
   1 = zoom + 1
Else
        If 1 + zoom > sizey Then 1 = sizey - zoom
End If

zoomx = c: zoomy = 1
frmzoom.Show
picture2.Cls
End Sub
```

A2.2 list.frm

```
Sub Form_Load ( )
Dim t As Integer

flag = 0
For t = 1 To numfile
        list1.AddItem pathin (t)
Next t

End Sub

**********************************************************************

Sub CmdOk_Click ( )
Unload frmlist
End Sub

**********************************************************************

Sub cmdremove_Click ( )
Dim t As Integer
Dim index As Integer

index = list1.ListIndex
If index = -1 Then index = 0
If numfile > 0 Then
        list1.RemoveItem index
        For t = index +  To numfile - 1
                pathin (t) = pathin (t + 1)
                pathout (t) = pathout (t + 1)
        Next t
        numfile = numfile - 1
End If
End Sub

**********************************************************************

Sub Form_Load ( )
Dim t As Integer

flag = 0
```

```
For t = 1 To numfile
        list1.AddItem pathin (t)
Next t

End Sub
```

A2.3 loadpicture.frm

```
declarations
Option Explicit

**********************************************************************

Sub Form_Load ( )
Dim s As String
If pathin (0) < > "" Then
        s = pathin (0)
        Do While (Right (s, 1) < > "\")
                s = Left (s, Len (s) - 1)
        Loop
        file1.Path = s
        dir1.Path = s
        drive1.drive = drive
End If
End Sub

**********************************************************************

Sub Command1_Click ( )
Dim flag As Integer

flag = 0
On Error GoTo CheckError
        If Right (file1.Path, 1) < > "\" Then
                frmmain.Label1.Caption = file1.Path & "\" & file1.FileName
        Else
                frmmain.Label1.Caption = file1.Path & file1.FileName
        End If
                frmmain.Image1.Picture = LoadPicture (frmmain.Label1.Caption)
        If flag = 0 Then
                numfile = numfile + 1
                pathin (numfile) = frmmain.Label1.Caption
                pathin (0) = pathin (numfile) : drive = drive1.drive
```

```
                pathout (numfile) = Left (pathin (numfile),
Len (pathin (numfile)) - 4) & "#.bmp"
                frmmain.Text1.Visible = True
                frmmain.Text1.Text = pathout (numfile)
                Unload frmlist
                frmlist.Show
        Unload frmload
        End If
        Exit Sub
CheckError:
        Const ERR_FILENOTFOUND = 53
If (Err = ERR_FILENOTFOUND) Then flag = 1: Resume Next
End Sub

**********************************************************************

Sub Command2_Click ( )
Unload frmload
End Sub

**********************************************************************

Sub Dir1_Change ( )
file1.Path - dir1.Path
End Sub

**********************************************************************

Sub Drive1_Change ( )
dir1.Path = drive1.drive
End Sub

**********************************************************************

Sub File1_DblClick ( )
        If Right (file1.Path, 1) < > "\" Then
                frmmain.Label1.Caption = file1.Path & "\" & file1.FileName
        Else
                frmmain.Label1.Caption = file1.Path & file1.FileName
        End If
                frmmain.Image1.Picture = LoadPicture (frmmain.Label1.Caption)

        numfile = numfile + 1
```

```
        pathin (numfile) = frmmain.Label1.Caption
        pathin (0) = pathin (numfile): drive = drive1.drive
        pathout (numfile) = Left (pathin (numfile), Len (pathin (numfile)) - 4) & "#.bmp"
        frmmain.Text1.Visible = True
        frmmain.Label2.Visible = True
        frmmain.Text1.Text = pathout (numfile)
        Unload frmlist
        frmlist.Show
        Unload frmload
End Sub
*****************************************************************

Sub Form_Load ( )
Dim d As String

If pathin (0) < > "" Then
        s = pathin (0)
        Do While (Right (s, 1) < > "\"
                s = Left (s, Len (s) - 1)
        Loop
        file1.Path = s
        dir1.Path = s
        drive1.drive = drive
End If
End Sub
```

A2.4 main.frm

```
declarations
Option Explicit

*****************************************************************

Sub Form_Load ( )

        ScrX = Screen.TwipsPerPixelX
        ScrY = Screen.TwipsPerPixelY
        numfile = 0
        pathin (0) = ""
        fracflag = 0
        thescale = .00826
        limcut = 10
```

```
End Sub
**********************************************************************

Sub mnuFileItem_Click (index As Integer)

        flag = 1
        Select Case Index
                Case 0
                        frmload.Show
                Case 1
                        frmlist.Show
                Case 2
                        End
                End Select
End Sub

**********************************************************************

Sub mnuRunItem_Click (index As Integer)
        Select Case Index
                Case 0
                        frmoption.Show
                Case 1
                        frmrun.Show
                End Select
End Sub

**********************************************************************

Sub Text1_Change ( )
pathout (numfile) = text1.Txt
End Sub
```

A2.5 option.frm

```
declarations
Option Explicit

**********************************************************************

Sub Check1_Click ( )
        fracflag = check1.Value
```

```
End Sub
**********************************************************************

Sub cmddefault_Click ( )
        fracflag = 0
        thescale = .00826
        limcut = 10
        check1.Value = 0
        text1.Text = thescale
        text2.Text = limcut
End Sub

**********************************************************************

Sub Command1_Click ( )
Unload frmoption
End Sub

**********************************************************************

Sub Form_Load ( )
        If fracflag = 0 Then
                check1.Value = 0
        Else
                check1.Value = 1
        End If
        text1.Text = thescale
        text2.Text = limcut
End Sub

**********************************************************************

Sub Text1_Change ( )
If IsNumeric (text1.Text) Then thescale = text1.Text
End Sub

**********************************************************************

Sub Text2_Change ( )
If IsNumeric (text 2.Text) Then limcut = text2.Text
End Sub
```

A2.6 run1.frm

A2.6.1 general

```
declarations
Option Explicit

Dim filentext
Dim pathtext As String
Dim tmem As Integer

DoAll

Sub DoAll (ByVal pathin As String, ByVal pathout As String)
Dim tot As Single, av As Single, va As Single, fracd As Single
Dim bra0 As Single, bra1 As Single, bra2 As Single, bra3 As Single
Dim num0 As Single, num1 As Integer, num2 As Integer, num3 As Integer
Dim t As Integer, c As Integer
Dim s As String

c = 1: s = "a"
Do While Left (s, 1) < > "\"
        s = Right (pathin, c)
        c = c + 1
Loop
s = Right (s, Len (s) - 1)
PrintIt filentext, "analysis of file" & s
PrintIt filentext, ""
picture2.Print "analysis of file" & s

'loading
picture1.Cls
picture1.Print "start loading"

LoadPict (pathin)
picture3.Width = sizex * ScrX: picture3.Height = sizey * ScrY
picture3.Picture = LoadPicture (pathin)
picture1.Cls

'skeleton
picture1.Print "computing skeleton"
Skeleton
```

```
picture1.Cls
picture1.Print "erasing components of less than " & limcut & "pixels"
Components (1)
Cutsmall
PrintIt Filentext, "connected components:" & ncomp

'fractal dimension
If fracflag = 1 Then
        picture1.Cls
        picture1.Print "computing fractal dimension"
        fracd = fracDim ( )
        PrintIt flientext, "fractal dimension: " & fracd
        picture2.Print "fractal dimension:" & fracd
End If

'compute length
picture1.Cls
picture1.Print "computing length of branches"
Branches tot, av, va, bra0, bra1, bra2, bra3, num0, num1, num2, num3
picture1.Cls
PrintIt filentext, "number of intersections:" & ncompinter
PrintIt filentext, "number of hyphae: " & ncomp
PrintIt filentext, "total length of hyphae:" & tot & "mm"
picture2.Print "total length: " & tot & "mm"
PrintIt filentext, "average length:" & av & "mm"
PrintIt filentext, "strd dev of length:"  & Sqr (va) & "mm"
PrintIt filentext, ""
PrintIt filentext, "number of branches:" & num2
PrintIt filentext, "total length of branches:" & bra2
PrintIt filentext, "number of inner hyphae:" & num3
PrintIt filentext, "total length of inner hyphae:" & bra3
PrintIt filentext, "number of isolated open hyphae:" & num1
PrintIt filentext, "total length of isolated open hyphae:" & bra1
PrintIt filentext, "number of loops:" & num0
PrintIt filentext, "total length of loops:" & bra0
PrintIt filentext, ""
PrintIt filentext, ""
PrintIt filentext, ""
picture2.Print Chr$ (13)

'save picture
picture1.Cls
picture1.Print "saving picture...please wait."
```

```
SavePict (pathout)
picture1.Cls
End Sub

DoFrac

Sub DoFrac (ByVal pathin As String, ByVal pathout As String)
Dim tot As Single, av As Single, va As Single, fracs As Single
Dim t As Integer, c As Integer
Dim s As String

c = 1: s = "a"
Do While Left (s, 1) < > "\"
        s = Right (pathin, c)
        c = c + 1
Loop
s = Right (s, Len (s) - 1)
PrintIt filentext, "analysis of file" & s
picture2.Print "analysis of file" & s

'loading
picture1.Print "start loading"
LoadPict (pathin)
picture3.Width = sizex * ScrX: picture3.Height = sizey * ScrY
picture3.Picture = LoadPicture (pathin)
picture1.Cls

'fractal dimension

        picture1.Cls
        picture1.Print "computing fractal dimension"
        fracd = FracDim ( )
        PrintIt filentext, "fractal dimension:" & fracd
        picture2.Print "fractal dimension:" & fracd

End Sub

Others

Sub Paint (ByVal t As Integer, ByVal col As Long)

Dim i As Integer, j As Integer
Aim a1 As Integer, b1 As Integer
```

```
Dim c As Integer, l As Integer
Dim x As Integer, y As Integer
Dim ex As Integer

        c = 0: l = 0
        i = ends (t, 1): j = ends (t, 2)
        Do
        ex = 0; x = 0; y = 0
        For a1 = -1 To 1
                For b1 = -1 To 1
                If ((1 + a1 < > c Or j + b1 < > 1) And (a1 < > 0 Or b1 < > 0)) Then
                        If pic (i + a1, j + b1) < > 0 Then
                                x = 1 + a1: y = j + b1
                                If g (x, y) = t Then
                                        ex = 1
                                        picture3.PSet (i - 1, j - 1), col
                                        c = i: l = j
                                        i = x: j = y
                                        Exit For
                                End If
                        End If
                End If
                Next b1
                If ex = 1 Then Exit For
Next a1
Loop While ex = 1
'If (x = 0 And y = 0) Then
'       ends (t, 3) = i: ends (t, 4) = j
' Else
        '       ends (t, 3) = x: ends (t, 4) = y
        ' End If
End Sub

**********************************************************************

Sub PrintIt (ByVal fileindex As Integer, ByVal s As String)

Print #fileindex, s
results = results & s & Chr (13) & Chr (10)
End Sub
```

A2.6.2 Commands

```
Sub CmdEdit_Click ( )
        frmedit. Show
        frmresults.text1 = results
        frmresults. Show
End Sub

*************************************************************************

Sub CmdQuit_Click ( )
        Unload Me
End Sub

*************************************************************************

Sub CmdStart_Click ( )
Dim t As Integer

' check that there are files to analyse
If numfile = 0 Then Picture1.Cls:
                picture1.Print "The file list is empty!": Exit Sub

sqr2 = Sqr (2)

'give path to store results file
pathtext = pathin (numfile)
Do While Right (pathtext, 1) < > "\"
        pathtext = Left (pathtext, Len (pathtext) - 1)
Loop
pathtext = pathtext & "result.txt"

'open the textfile
filentext = Freefile
Open pathtext For Append As filentrxt Len = 1

picture2.Cls
'analyse all the pictures
For t = 1 To numfile
        DoAll pathin (t), pathout (t)
Next t

Close filentext
Unload frmzoom
Unload frmedit
```

```
pathin (0) = pathin (numfile)
pathout (0) = pathout (numfile)
CmdEdit. Visible = True

End Sub

**********************************************************************

Sub Form_Load ( )
picture3.ScaleMode = 3
tmem = 0
End Sub
```

A2.7 zoom.frm

```
declarations
Option Explicit

Dim side As Integer

**********************************************************************

Sub Drawzoom ( )
Dim c As Integer, 1 As Integer, col As Long, p As Integer

For c = 0 To 2 * zoom
        For 1 = 0 To 2 * zoom
        p = pic(zoomx + c - zoom, zoomy + 1 - zoom)
        If p > 0 Then
                col = 0
        ElseIf p = 2 Then
                col = RGB (0, 0, 255)
        Else
                col = RGB (255, 0, 0)
        End If
  Picture1.Line (c * side, 1 * side) - ((c + 1) * side, (l + 1) * side), col, BF
  End If
  Picture1.Line (c * side, 1 * side) - ((c + 1) * side,
(l + 1) * side), RGB (180, 180, 180), B
  Next l
Next c
End Sub
```

```
**********************************************************************
Sub CmdOk_Click ( )
Unload Me
End Sub

**********************************************************************

Sub Form_Load

side = 10
Picture1.ScaleMode = 3
Picture1.Width = ((zoom * 2 + 1) * side + 2) * ScrX
Picture1.Height = ((zoom * 2 + 1) * side + 2) * ScrY
Drawzoom
End Sub

**********************************************************************

Sub Picture1_MouseDown (Button As Integer, Shift As Integer,
  X As Single, Y As Single)
Dim c As Integer, l As Integer
Dim col As Long

c = X \ side: l = Y \ side
If OptWrite.Value = True Then
        col = 0: pic (zoomx - zoom + c, zoomy - zoom + l) = 1
Else
        col = RGB (255, 255, 255): pic (zoomx - zoom + c, zoomy - zoom + l) = 0
End If
Picture1.Line (c * side, l * side) - ((c + 1) * side, (l + 1) * side ), col, BF
frmedit. Picture1.PSet (zoomx - zoom + c - 1, zoomy - zoom + l - 1), col

Picture1.Line (c * side, l * side) - ((c + 1) * side, (l + 1) * side),
  RGB (180, 180, 180), B
End Sub
```

A2.8 general procedures

A2.8.1 Declarations

```
Option Explicit

Type pixel
```

```
        x As Integer
        y As Integer
End Type

Global pic ( ) As Integer
Global g ( ) As Integer

Global sizecomp (10000) As Integer
Global ncomp As Integer

Global sizex As Long, sizexr As Long, sizey As Long
Global sizeo As Long, sizep As Long, sizef As Long
Global flag As Integer
Global lmax As Integer
Global limcut As Integer
Global branch (10000) As Integer, ends (10000, 1 To 4) As Integer
Global onepoint (10000, 1 To 2) As Integer
Global ninter As Integer, interpoint (10000) As pixel
Global nend As Integer, endpoint (10000) As pixel

Global ncompinter
Global length (10000) As Single
Global sqr2 As Single

'used for the images
Global num As Long
Global filenum As Integer
Global begi As String, begi2 As String

'keep track of the files
Global fracflag As Integer
Global pathin (100) As String
Global pathout (100) As String
Global drive As String
Global numfile As Integer

'to display the results
Global thescale As Single
Global results As String
Global ScrX As Single
Global ScrY As Single

Global zoomx As Integer, zoomy As Integer, zoom As Integer
```

A2.8.2 bran

```
Function Bran (ByVal t As Integer) As Single

Dim i As Integer, j As Integer
Dim a1 As Integer, b1 As Integer
Dim c As Integer, l As Integer
Dim startx As Integer, starty As Integer
Dim x As Integer, y As Integer
Dim ex As Integer
Dim tot As Single

        tot = 0: c = 0: l = 0
        i = ends (t, 1): j = ends (t, 2)
        If branch (t) = 0 Then i = onepoint (t, 1): j = onepoint (t, 2)
        startx = i: starty = j
        Do
        ex = 0: x = 0: y = 0
        For a1 = -1 To 1
                For b1 = -1 To1
                If ((i + a1 < > c Or j + b1 < > l) And (a1 < > 0 Or b1 < > 0)
And (i + a1 < > startx Or j + b1 < > starty) Then
                        If pic (i + a1, j + b1 < > 0 Then
                                x = i + a1: y = j + b1
                                If g(x, y) = t Then
                                        ex = 1
                                        If (a1 = 0 Or b1 = 0) Then
                                                tot = tot + 1
                                        Else
                                                tot = tot + sqr2
                                        End If
                                        c = i: l = j
                                        i = x: j = y
                                        Exit For
                                End If
                        End If
                End If
                Next b1
                If ex = 1 Then Exit For
        Next a1
        Loop While ex = 1
        If (x = 0 And y = 0) Then
```

```
            ends (t, 3) = x: ends (t, 4) = y
            If (x = i Or y = j) Then
                tot = tot + 1
            Else
                tot = tot + sqr2
            End If
            End If
        Bran = tot * thescale / 1.05
        End Function
```

A2.8.3 Branches

```
Sub Branches (totl As Single, average As Single, variance As Single,
  bra0 As Single, bra1 As Single, bra2 As Single,
  bra3 As Single, num0 As Single, num1 As Integer,
  num2 As Integer, num3 As Integer)

Dim 1 As Integer, j As Integer
Dim c As Integer, l As Integer
Dim p As pixel
Dim s As Integer, t As Integer

'find intersection and end points
ninter = 0: nend = 0
For i = 1 To sizex
        For j = 1 To sizey
        If pic (i, j) = 1 Then
                s = pic (i - 1, j - 1) + pic (i, j - 1) + pic (i + 1, j - 1) +
pic (i - 1, j) + pic (i + 1, j) + pic (i - 1, j + 1) + pic (i, j + 1) + pic (i + 1, j + 1)
                If s < 2 Then
                    nend = nend + 1
                    endpoint (nend). x = 1: endpoint (nend). y = j
                End If
                If s > 2 Then
                    ninter = ninter + 1
                    interpoint (ninter).x = i: interpoint (ninter).y + j
                End If
        End If
        Next j
Next i

'remove intersection points
For t = 1 To ninter
```

```
        pic (interpoint (t).x, interpoint (t).y) = 2
Next t

'calculate connected components of intersections
Components (2)
ncompinter = ncomp

'calculate connected components of branches
Components (1)

'remove small components
'Cutsmallb

'initialise branch
'branch describes the links between the hypha and the network:
'0 = isolated loop, 1 = isolated string, 2 = branch, 3 = joining two
'intersection points
For i = 1 To ncomp
        branch (i) = 0
Next i

'specify the ends of the branches
For t = 1 To nend
        i = endpoint (t).x: j = endpoint (t).y
        For c = -1 To 1
                For l = -1 To 1
                        If pic (i + c, j + l) = 1 Then
                                ends (g (i + c, j + l), 1) = i
                                ends (g (i + c, j + l), 2) = j
                                branch (g (i + c, j + l)) = 1
                        End If
                Next l
        Next c
Next t

For t = 1 to ninter
    i = interpoint (t).x: j = interpoint (t).y
                For c = -1 To 1
                        For l = -1 To 1
                                If pic (i + c, j + l) = 1 Then
                                        ends (g (i + c, j + l), 1) = i
                                        ends (g (i + c, j + l), 2) = j
                                        If branch (g (i + c, j + l)) = 1
```

```
        Or branch (g (i + c, j + l)) = 2 Then
                                        branch (g (i + c, j + l)) = 2
                                Else
                                        branch (g (i + c, j + 1)) = 3
                                End If
                        End If
                Next l
        Next c
Next t

'restore intersection points
'For t = 1 To ninter
'pic (interpoint (t). x, interoint (t).y) = 1
'Next t

'compute length of branches
totl = 0
bra0: bra1 = 0: bra2 = 0: bra 3 = 0
num0: num1 = 0: num2 = 0: num3 = 0
For t = 1 To ncomp
        length (t) = Bran (t)
        Select Case branch (t)
        Case 0
                bra0 = bra0 + length (t): num0 = num0 + 1
        Case 1
                bra1 = bra1 + length (t): num1 = num1 + 1
        Case 2
                bra2 = bra2 + length (t): num2 = num2 + 1
        Case 3
                bra3 = bra3 + length (t): num3 = num3 + 1
        End Select
        totl = totl + length (t)
Next t

'change value of end points
For t = 1 To nend
        pic (endpoint (t).x, endpoint (t).y) = 3
Next t

average = totl / ncomp: variance = 0
For t = 1 To ncomp
        variance = variance + (length (t) - average) * (length (t) - average)
Next t
```

```
variance = variance / ncomp
End Sub
```

A2.8.4 Components

```
Dim i As Integer, j As Integer, k As Integer, l As Integer, m As Long
Dim a As Long, b As Long, c As Long, d As Long, s As Integer
Static w (10000) As Integer
Static h (10000) As Integer
Static v (10000) As Integer
Static u (10000) As Integer
Dim flagb As Integer, ncomp1 As Integer

'initialise the data
ncomp = 0
For i = 1 To sizex
        For j = 1 To sizey
        g(i, j) = 0
        Next j
Next i
For i = 0 To 10000
        h (i) = i
        sizecomp (i) = 0
Next i

'raster scan
For j = 1 To sizey
        For i = 1 To sizex
        If pic (i, j) = col Then
                s = 0
                If pic (i-1, j-1) = col Then s = s + 1
                If pic (i, j-1) = col Then s = s + 1
                If pic (i + 1, j - 1) = col Then s = s + 1
                If pic (i-1, j) = col Then s = s + 1
                If (s = 0) Then
                ncomp = ncomp + 1: g (i, j) = ncomp: sizecomp (ncomp) = 1
                onepoint (ncomp, 1) = i: onepoint (ncomp, 2) = j
                ElseIf (s = 1) Then
                k = g (i - 1, j - 1) + g (i, j - 1) + g (i + 1, j - 1) + g (i - 1, j)
                g (i, j) = k: sizecomp (k) = sizecomp (k) + 1
                Else
                a = h (g (i - 1, j - 1)): b = h (g (i, j-1)):
c = h (g (i +1, j-1)): d = h (g (i - 1, j))
```

```
                m = (a + b + c + d) \ s
                If (a * (a-m) = 0 And b * (b - m) = 0 And
c * (c - m) = 0 And d * (d - m) = 0) Then
                g (i, j) = m: sizecomp (m) = sizecomp (m) + 1
                        Else
                                k = m
                                If k > a And a > 0 Then k = a
                                If k > b And b > 0 Then k = b
                                If k > c And c > 0 Then k = c
                                If k > d And d > 0 Then k = d
                                g (i, j) = k
                                sizecomp (k) = sizecomp (k) + 1
                                For l = 1 To ncomp
                                        If (h (l) = a Or h (l) = b Or h (l) = c Or
h (l) = d) Then h (l) = k
                                Next l
                        End If
                End If
        End If
        Next i
Next j

'unification
ncomp1 = ncomp
For l = 1 To ncomp
        If h (l) < l Then sizecomp (h (l)) + sizecomp (h (l)) + sizecomp (l)
Next l
i = 0
v (0) = 0: u (0) = 0
For l = 1 To ncomp
        v (l) = l: u (l) = l
        If h (l) = l Then
        i = i + 1
        sizecomp (i) = sizecomp (l)
        w (h (l)) = i
        End If
Next l
ncomp = i
Do
flagb = 0
For l = 2 To ncomp
        If sizecomp (l) > sizecomp (l -1) Then
        i = sizecomp (l - 1): sizecomp (l - 1) = sizecomp (l): sizecomp (l) = l
```

```
        i = v (u (l - 1)): v (u (l - 1)) = v(u (l)): v (u (l)) = 1
        i = u (l - 1): u (l - 1) = u (1): u (l) = i
        flagb = 1
        End If
Next l
Loop While flagb = 1

For l = 1 To ncomp l
        onepoint (v (w (h (l))), 1) = onepoint (l, 1)
        onepoint (v (w (h (l))), 2) = onepoint (l, 2)
Next l

For i = 1 To sizex
        For j = 1 To sizey
        g (i, j) = v (w (h (g (i, j))))
        Next j
Next i
End Sub
```

A2.8.5 CountSquares

```
Function CountSquares (ByVal r As Integer, ByVal bord As Integer) As Long

Dim i As Integer, j As Integer
Dim c As Integer, l As Integer
Dim total As Long, temp As Integer

total = 0

For i = bord + 1 To bord + sizex - r Step r
        For j = bord + 1 To bord + sizey - r Step r
        temp = 0
                For c = i To i + r
                        For l = j To j + r
                        If g (c, l) = 1 Then total = total + 1: temp = 1: Exit For
                        Next l
                        If temp = 1 Then Exit For
                Next c
        Next j
Next i
'For i = bord + 1 To bord + sizex
'       For j = bord + 1 To bord + sizey
'       If g (i, j) = 1 Then
```

```
'                   temp = 0
'                   For c = 1 - r To i + r
'                           For l = j - r To j + r
'                                   temp = temp + g (c, l)
'                           Next l
'                   Next c
'                   total = total + temp
'           End If
'           Next j
'Next i
CountSquares = total
End Function
```

A2.8.6 CutSmall

```
Sub Cutsmall ( )
Dim i As Integer, j As Integer

For i = 1 To sizex
        For j = 1 To sizey
                If sizecomp (g (i, j)) <= limcut Then pic (i, j) = 0
        Next j
Next i

i = 1
Do Until (sizecomp (i) <= limcut Or i > ncomp)
i = i + 1
Loop
ncomp = i - 1
End Sub
```

A2.8.7 CutSmall

```
Sub Cutsmallb ( )
Dim i As Integer, j As Integer
Dim t As Integer, n As Integer, tot As Integer
Static v (10000) As Integer

limcut = 3
For t = 1 To nend
        i = endpoint (t). x: j = endpoint (t). y
        If sizecomp (g (i, j)) <= limcut Then sizecomp (g (i, j)) = 0
Next t
```

```
'initialise permutation v
For t = 0 To ncomp
        v (t) = t
Next t

'Calculate permutation v
n = 0: tot = ncomp
For t = 1 To tot
        n = n + 1
        If sizecomp (t) = 0 Then n = n - 1: ncomp = ncomp - 1
        v (t) = n
Next t

'erase small components from grid
For i = 1 To sizex
        For j = 1 To sizey
                If pic (i, j) = 1 Then
                        If sizecomp (g (i, j)) = 0 Then
                                g (i, j) = 0: pic (i, j) = 0
                        Else
                                g (i, j) = v (g (i, j))
                        End If
                End If
        Next j
Next i

'modify array sizecomp ( )
n = 0: t = 0
Do
        t = t + 1: n = n + 1
        Do While (sizecomp (t) = 0 And t < tot)
                t = t + 1
        Loop
        sizecomp (n) = sizecomp (t)
Loop While t < tot
End Sub
```

A2.8.8 FracDim

```
Function FracDim ( ) As Single

Static r ( 1 To 20) As Single, s (1 To 20 ) As Single
```

```
Dim nmes As Integer
Dim a As Single, b As Single, c As Single, d As Single
Dim t As Integer, i As Integer, j As Integer, x As Integer, y As Integer
Dim bord As Integer
Dim factor As Single

nmes = 3
r (1) = 1: r (2) = 2: r (3) = 3: r (4) = 4: r (5) = 5
bord = r (nmes)

'Copy pic in g in order to avoid boundary problems
ReDim g (sizex + bord * 2, sizey + bord * 2)

For i = 1 To sizex + 2 * bord
        x = i
        If x < bord + 1 Then x = 2 * bord - x + 1
        If x > (sizex + bord) Then x = 2 * (sizex + bord) - x + 1
        For j = 1 To sizey + 2 * bord
                y = j
                If y < bord + 1 Then y = 2 * bord - y + 1
                If y > (sizey + bord) Then y = 2 * (sizey + bord) - y + 1
                g (i, j) = 0
                If pic (x-bord, y - bord) > 0 Then g (i, j) = 1
        Next j
Next i
For t = 1 To nmes
        s (t) = CountSquares (r (t), bord)
Next t

For t = 1 To nmes
        s (t) = Log (s (t))
        r (t) = Log (r (t))
Next t

a = 0: b = 0: c = 0: d = 0
For t = 1 To nmes
    a = a + r (t)
    b = b + s (t)
    c = c + r (t) * s (t)
    d = d + r (t) * r (t)
Next t

FracDim = - (nmes * c - a * b) / (nmes * d - a * a)
```

```
factor = (b * d - a * c) / (nmes * d - a * a)
End Function
```

A2.8.9 GetLong

```
Function GetLong (x As Long) As Long
Dim sum As Long, z As Long
Dim y As String * 1
sum = 0
        For z = x + 3 To x Step - 1
        Get #filenum, z, y
        sum = sum * 256
        sum = sum + Asc (y)
        Next z
GetLong = sum
End Function
\subsection {InitGrid}
Sub InitGrid ( )
Dim i As Integer, j As Integer
sizex = 300: sizey = 300

ReDim pic (sizex + 1, sizey + 1)

For i = 0 To sizex + 1
        For j = 0 To sizey + 1
                pic (i, j) = 0
        Next j
Next i

For i = 30 To 150
        For j = 40 - 200
        pic (i, j) = 1
        Next j
Next i
End Sub
```

A2.8.10 LoadPict

```
Sub LoadPict (ByVal path As String)

Dim recordlen As Long
Dim x As String * 1
Static im ( ) As String * 1000
```

```
Dim c As Long, l As Long
Dim a As Long, b As Long, count As Long
Dim temp As String

Dim tmp, total As Long
Dim i As Integer

'Get some information about the file
        recordlen = Len (x)
        filenum = Freefile
Open path For Random As filenum Len = recordlen
        sizeo = GetLong (11)
        sizef = GetLong (3)
        sizep = GetLong (35)
        sizex = GetLong (19)
        sizey = GetLong (23)
Close filenum

ReDim pic (sizex + 1, sizey + 1)
ReDim g (sizex + 1, sizey + 1)
ReDim im (sizey + 1)

'sizexr is the first integer bigger than sizex and multiple of 4 (bytes)
sizexr = sizex + 3 - ((sizex + 3) Mod 4)

'Copy the file in several strings
        filenum = FreeFile
Open path For Binary As filenum
        begi = Input (sizeo, filenum)
        For 1 = 1 To sizey
                im (l) = Input (sizexr, filenum)
        Next l
Close filenum

'define all the offset for the thresholded picture
begi2 = ""

'enter the offset for bmp file

        num = 1
        'start with "BM"
        Writein (Asc ("B"))
        Writein (Asc ("M"))
```

```
'Size
total = 1078 + sizexr * sizey
tmp = total Mod 65536
Writein (tmp Mod 256): Writein (Int (tmp / 256))
tmp = Int (total / 65536)
Writein (tmp Mod 256): Writein (Int (tmp / 256))
'Reserved1 and Reserved2
Writein (0): Writein (0)
Writein (0): Writein (0)
'OffsetBits
Writein (54): Writein (4): Writein (0): Writein (0)
'Size
Writein (40): Writein (0): Writein (0): Writein (0)
'Width
Writein (sizex Mod 256); Writein (Int (sizex / 256)): Writein (0): Writein (0)
'Height
Writein (sizey Mod 256): Writein (Int (sizey / 256)): Writein (0): Writein (0)
'Planes
Writein (1): Writein (0)
'BitCount
Writein (8): Writein (0)
'Compression
Writein (0): Writein (0); Writein (0): Writein (0)
'SizeImage
total = sizexr * sizey
tmp = total Mod 65536
Writein (tmp Mod 256): Writein (Int (tmp / 256))
tmp = Int (total / 65536)
Writein (tmp Mod 256): Writein (Int (tmp / 256))
'XPelsPerMeter and YPelPer Meter
Writein (109): Writein (11): Writein (0): Writein (0)
Writein (109): Writein (11): Writein (0): Writein (0)
'ColorsUsed and ColoursImportant
Writein (0): Writein (1): Writein (0): Writein (0)
Writein (0): Writein (0): Writein (0): Writein (0)
'ColorTable
Writein (0): Writein (0): Writein (255): Writein (0)
Writein (255): Writein (0): Writein (0): Writein (0)
For i = 2 To 255
Writein (i): Writein (i): Writein (i): Writein (0)
Next i

'Copy the strings in an array
```

```
        For l = 1 To sizey
                temp = im (sizey + 1 - l)
                For c = 1 To sizex
                pic (c, l) = Asc (mid$ (temp, c, 1))
                If pic (c, l) > 150 Then pic (c, l) = 0 Else pic (c, l) = 1
                Next c
        Next l
'set the border values at 0
        For c = 0 To sizex + 1
        pic (c, 0) = 0: pic (c, sizey + 1) = 0
        Next c
        For l = 0 To sizey + 1
        pic (0, 1) = 0: pic (sizex + 1, l) = 0
Next l
End Sub
```

A2.8.11 Ordera

```
Function Ordera (By Val side As Integer) As Integer
Dim i As Integer, j As Integer
Dim s As Integer
Dim a1 As Integer, b1 As Integer
Dim tot As Integer, ord As Integer

ord = 0
For i = 1 To sizex
        For j = 1 To sizey
        g (i, j) = 0
        If pic (i, j) = 1 Then
                If ((side = 1 And pic (i + 1, j) = 1 Or
        (side = 2 And pic (i, j + 1) = 1) Or
        (side = 3 And pic (i-1, j) = 1) Or
        (side = 4 And pic (i, j - 1) = 1)) Then
                g (i, j) = 1
        Else
                tot = 0
                For a1 = -1 To 1
                        For b1 = -1 To 1
                        tot = tot + pic (i + a1, j + b1)
                        Next b1
                Next a1
                If tot = 1 Then
                        g  (i, j) = 0       'single pixel
```

```
                ElseIf tot = 2 Then
                        g  (i, j) = 1       'end pixel
                Else
                        s = 0
                        s = s + pic (i + 1, j + 1) * (1 - pic (i + 1, j)) *
(1 - pic (i, j + 1))
                s = s + pic (i + 1, j - 1) * (1 - pic (i + 1, j)) *
(1 - pic (i, j - 1))
                s = s + pic (i - 1, j - 1) * (1 - pic (i - 1, j)) *
(1 - pic (i, j - 1))
                s = s + pic (i - 1, j + 1) * (1 - pic ( i - 1, j)) *
(1 - pic (i, j + 1))
                If side = 1 Then s = s + pic (i, j + 1) *
(1 - pic (i - 1, j)) * pic (i, j - 1)
                If side = 2 Then s = s + pic (i + 1, j) *
(1 - pic (i, j - 1)) * pic (i - 1, j)
                        If side = 3 Then s = s + pic (i, j + 1) *
        (1 - pic (i + 1, j)) * pic (i, j - 1)
                        If side = 4 Then s = s + pic (i + 1, j) *
        (1 - pic (i, j + 1)) * pic (i -1, j)

                        If s > 0 Then
                                g (i, j) = 1        'keep the connection
                        Else
                                g (i, j) = 0: ord = 1
                        End If
                End If
        End If
        Next j
Next i
Ordera = ord
End Function
```

A2.8.12 Orderb

```
Function Orderb (ByVal side As Integer) As Integer
Dim i As Integer, j As Integer
Dim s As Integer
Dim a1 As Integer, b1 As Integer
Dim tot As Integer, ord As Integer

ord = 0
For i = 1 To sizex
```

```
        For j = 1 To sizey
        pic (i, j) = 0
        If g (i, j) = 1 Then
                If ((side = 1 And g (i + 1, j) = 1) Or (side = 2 And
g (i, j + 1) = 1) Or (side = 3 And g (i - 1, j) = 1 Or
(side = 4 And g (i, j - 1) = 1)) Then
                pic (i, j) = 1
        Else
                tot = 0
                For a1 = -1 To 1
                        For b1 = -1 To 1
                        tot = tot + g (i + a1, j + b1)
                        Next b1
                Next a1
                If tot = 1 Then
                        pic (i, j) = 0     'single pixel
                EsleIf  tot = 2 Then
                        pic (i, j) = 1     'end pixel
                Else
                        s = 0
                        s = s + g (i + 1, j + 1) * (1 - g (i + 1, j)) *
(1 - g (i, j + 1))
                        s = s + g (i + 1, j - 1) * (1 - g (i + 1, j)) *
(1 - g (i, j - 1))
                        s = s + g (i - 1, j - 1) * (1 - g (i - 1, j)) *
(1 - g (i, j - 1))
                        s = s + g (i - 1, j + 1) * (1 - g (i - 1, j)) *
(1 - g (i, j + 1))
                        If side = 1 Then s = s + g (i, j + 1) *
(1 - g (i - 1, j)) * g (i, j - 1)
                        If side = 2 Then s = s + g (i + 1, j) *
(1 - g (i, j - 1)) * g (i - 1, j)
                        If side = 3 Then s = s + g (i, j + 1) *
(1 - g (i + 1, j)) * g (i, j - 1)
                        If side = 4 Then s = s + g (i + 1, j) *
(1 - g (i, j + 1)) * g (i - 1, j)
                        If s > 0 Then
                                pic (i, j) = 1     'keep the connection
                        Else
                                pic (i, j) = 0: ord = 1
                        End If
                End If
         End If
```

```
End If
        Next j
Next i
Orderb = ord
End Function
```

A2.8.13 SavePicture

```
Sub SavePict (ByVal path As String)

Static im (1 To 1000) As String * 1000
Dim c As Long, l As Long
Dim a As Long, b As Long, count As Long
Dim temp As String, tem As Integer

'copy the array into strings
        For l = 1 To sizey
                For c = 1 To sizex
                tem = pic (c, sizey + 1 - l)
                If tem = 1 Then
                        Mid$ (im (l), c, 1) = Chr$ (2)
                ElseIf tem = 2 Then
                        Mid$ (im (l), c, 1) = Chr$ (1)
                ElseIf tem = 3 Then
                        Mid$ (im (l), c, 1) = Chr$ (0)
                Else
                        Mid$ (im (l), c, 1) = Chr$ (255)
                End If
                Next c
                For c = sizex + 1 To sizexr
                Mid$ (im (l), c, 1) = Chr$ (0)
                Next c
        Next l

'rewrite the strings into a file
        filenum = Freefile
        Open path For Binary As Filenum
        a = 5000 \ sizex
        b = sizey \ a: count = 1
        Put filenum, 1, begi2
        For l = 1 To b
                temp = ""
                For c = 1 To a
```

```
        temp = temp & (Left (im (count), sizexr))
                count = count + 1
                Next c
        Put filenum, , temp
        Next l
        For l = count to sizey
                temp = ""
                temp = Left (im (l), sizexr)
                Put filenum, , temp
        Next l
Close filenum
End Sub
\subsection {Skeleton}
Sub Skeleton ( )

Static flag (1 To 4) As Integer
Dim side As Integer, swap As Integer
Dim i As Integer, j As Integer

swap = 0: side = 3
flag (1) = 1: flag (2) = 1: flag (3) = 1: flag (4) = 1
Do
        If swap = 0 Then
                flag (side) = Ordera (side)
        Else
                flag (side) = Orderb (side)
        End If
        swap = 1 - swap: side = (side Mod 4) + 1
Loop While (flag (1) + flag (2) + flag (3) + flag (4) > 0
If swap = 1 Then
        For i = 1 To sizex
                For j = 1 To sizey
                pic (i, j) = g (i, j)
                Next j
        Next i
End If
End Sub
```

A2.8.14 WriteIn

```
Sub Writein (ByVal z As Integer)
begi2 = begi2 & Chr$ (z)
End Sub
```

Appendix 3

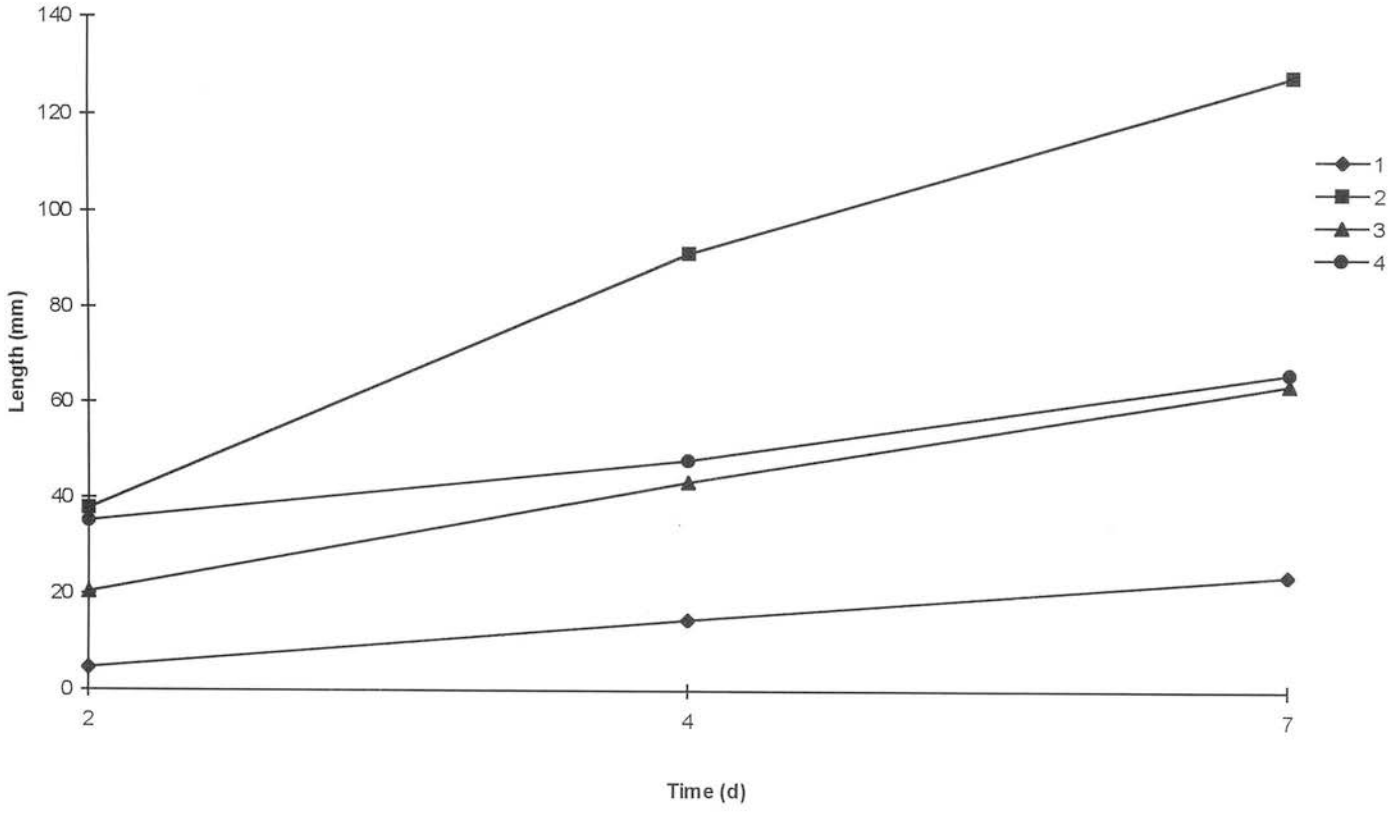

Figure A3.1a: Growth rates (mm/day) of individual replicates of the control

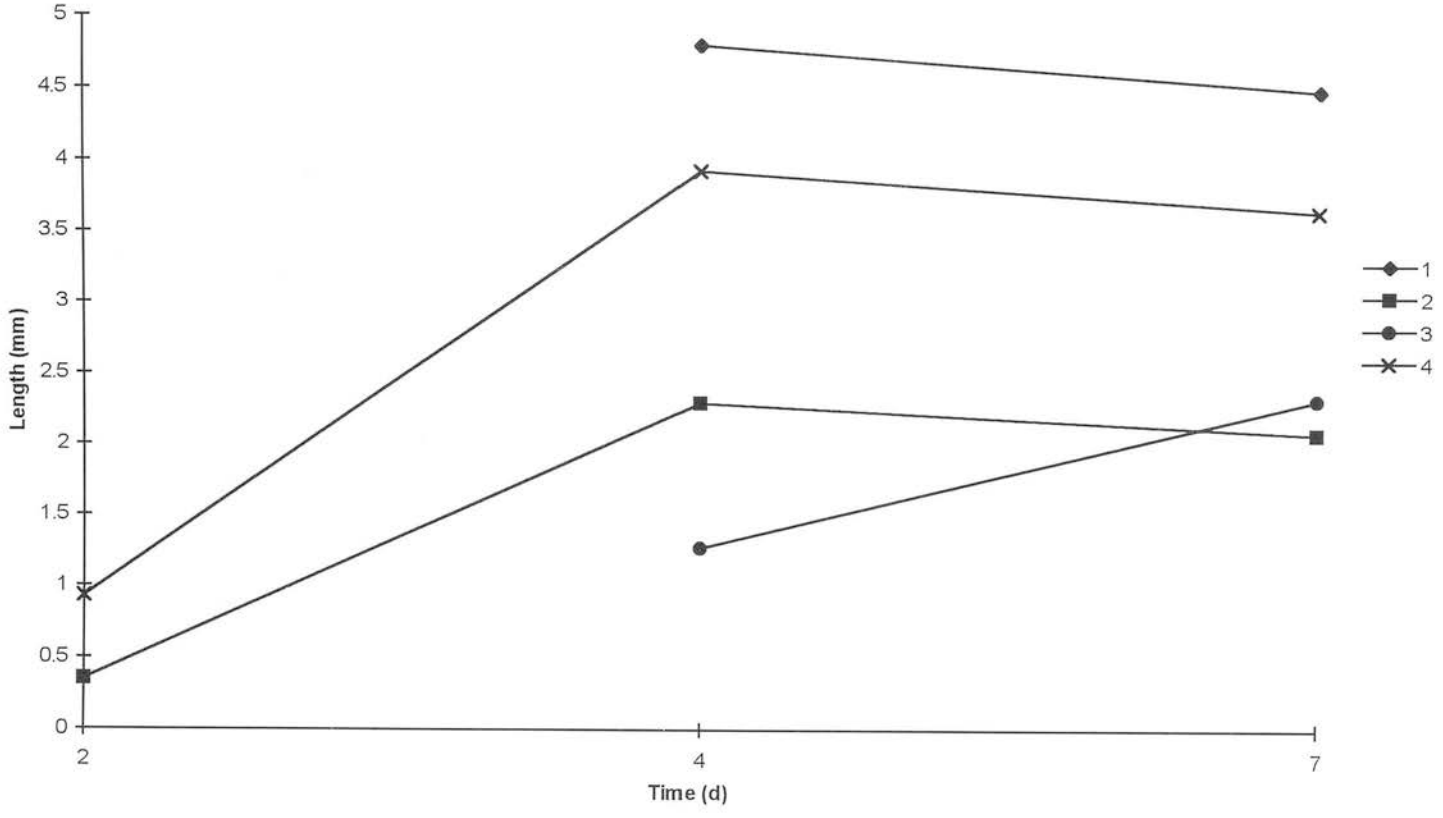

Figure A3.1b: Growth rates (mm/day) of individual replicates in the presence of host root exudates

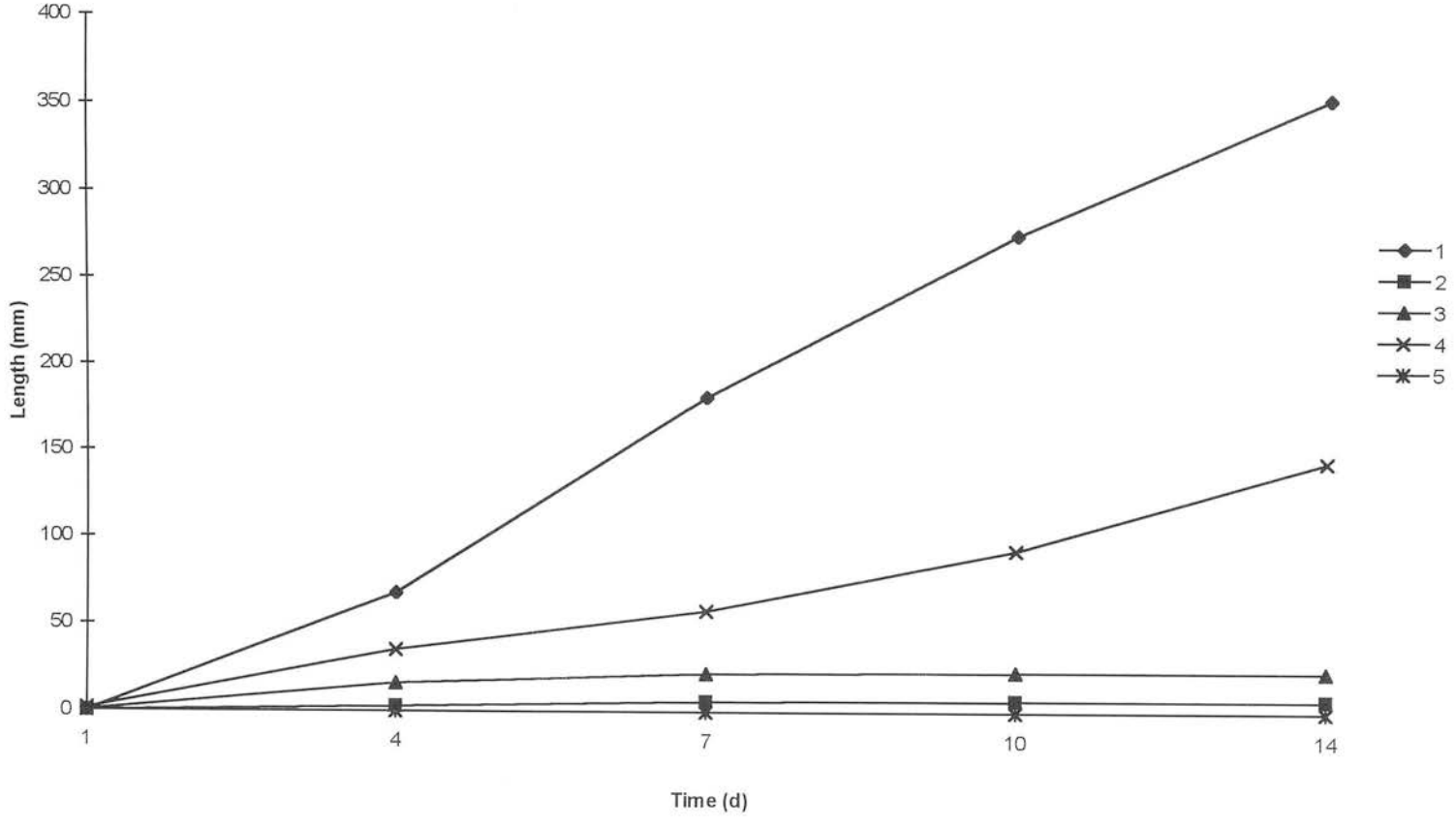

Figure A3.2a: Growth rates (mm/day) of individual replicates of the control

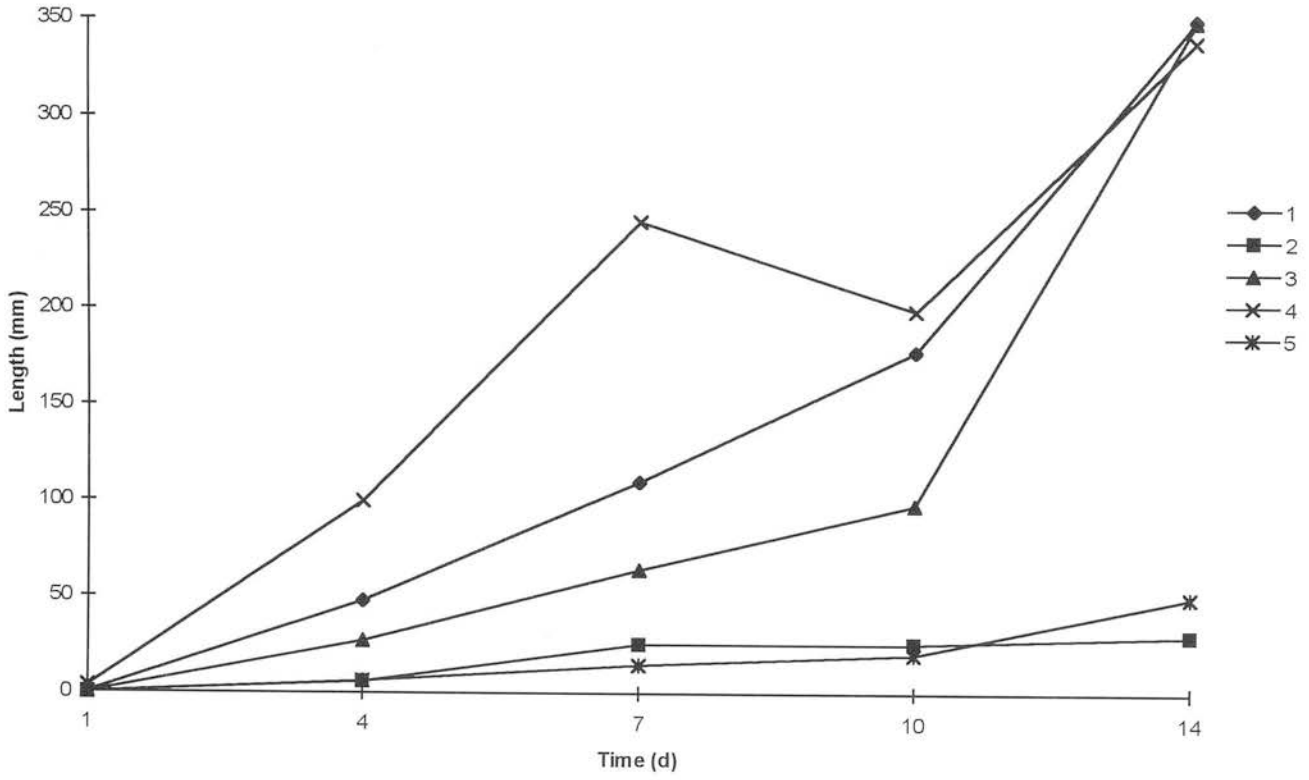

Figure A3.2b: Growth rates of individual replicates in the presence of host root exudates added at t=7 days

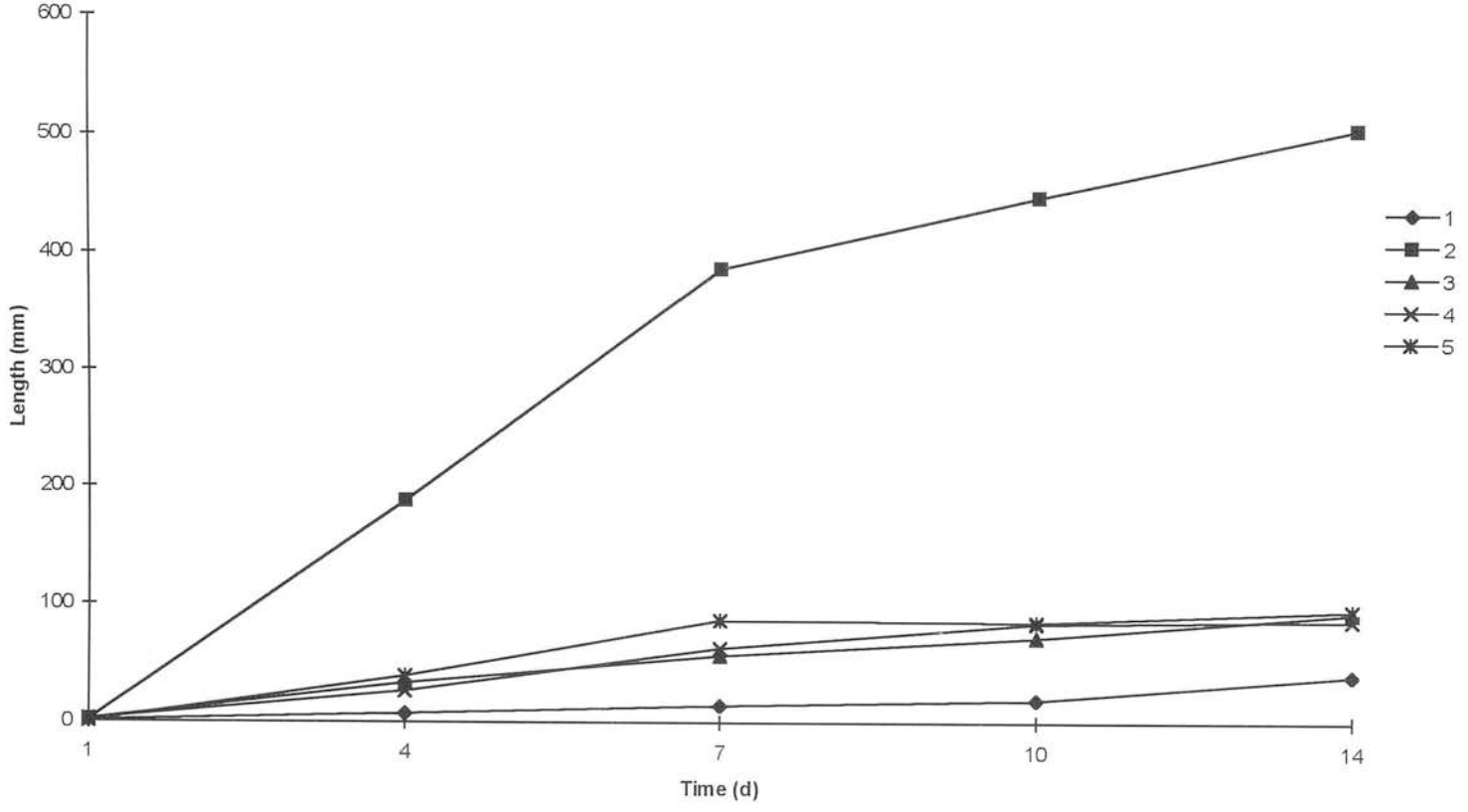

Figure A3.2c: Growth rates of individual replicates in the presence of non-host root exudates added at t=7 days

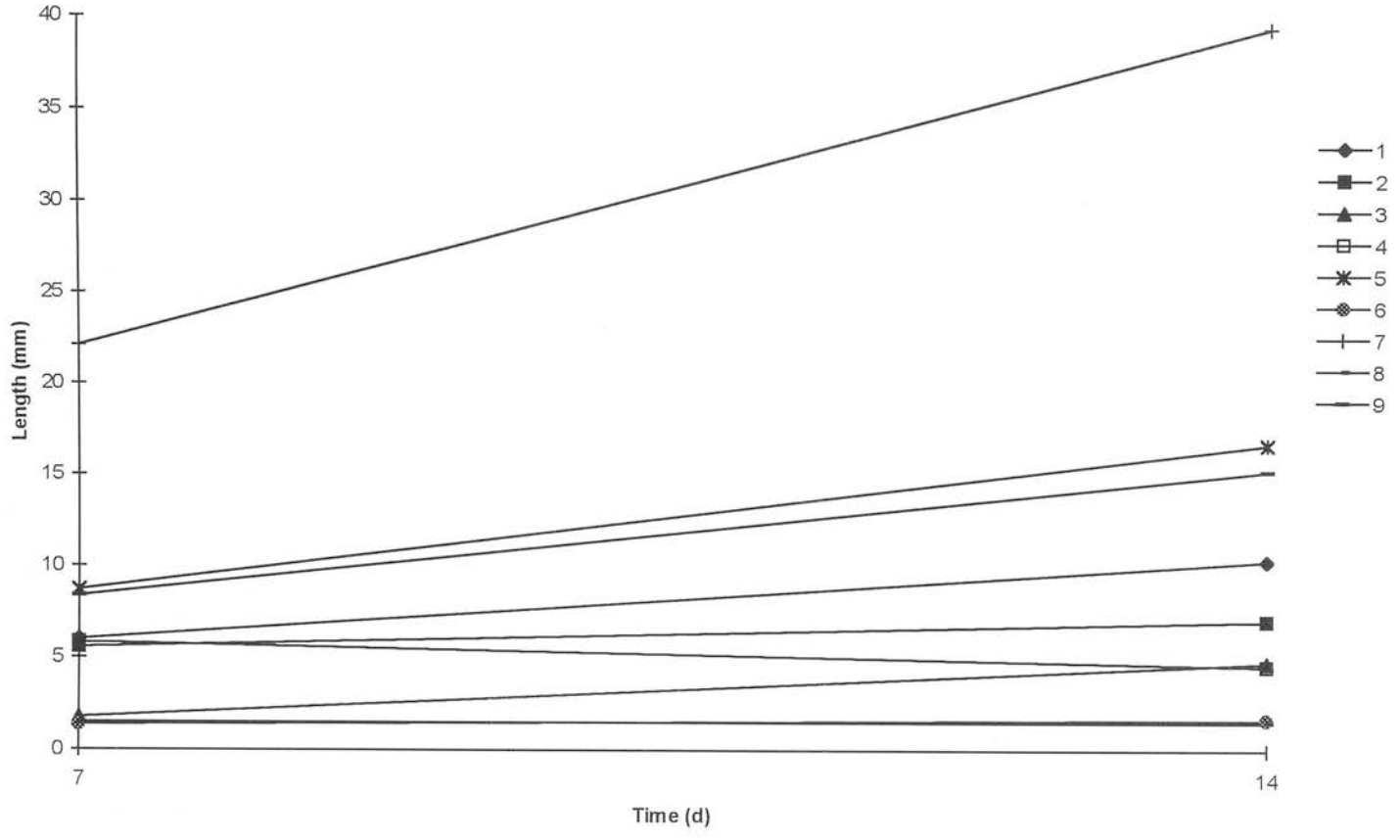

Figure A3.3a: Growth rates (mm/day) of individual replicates of the control

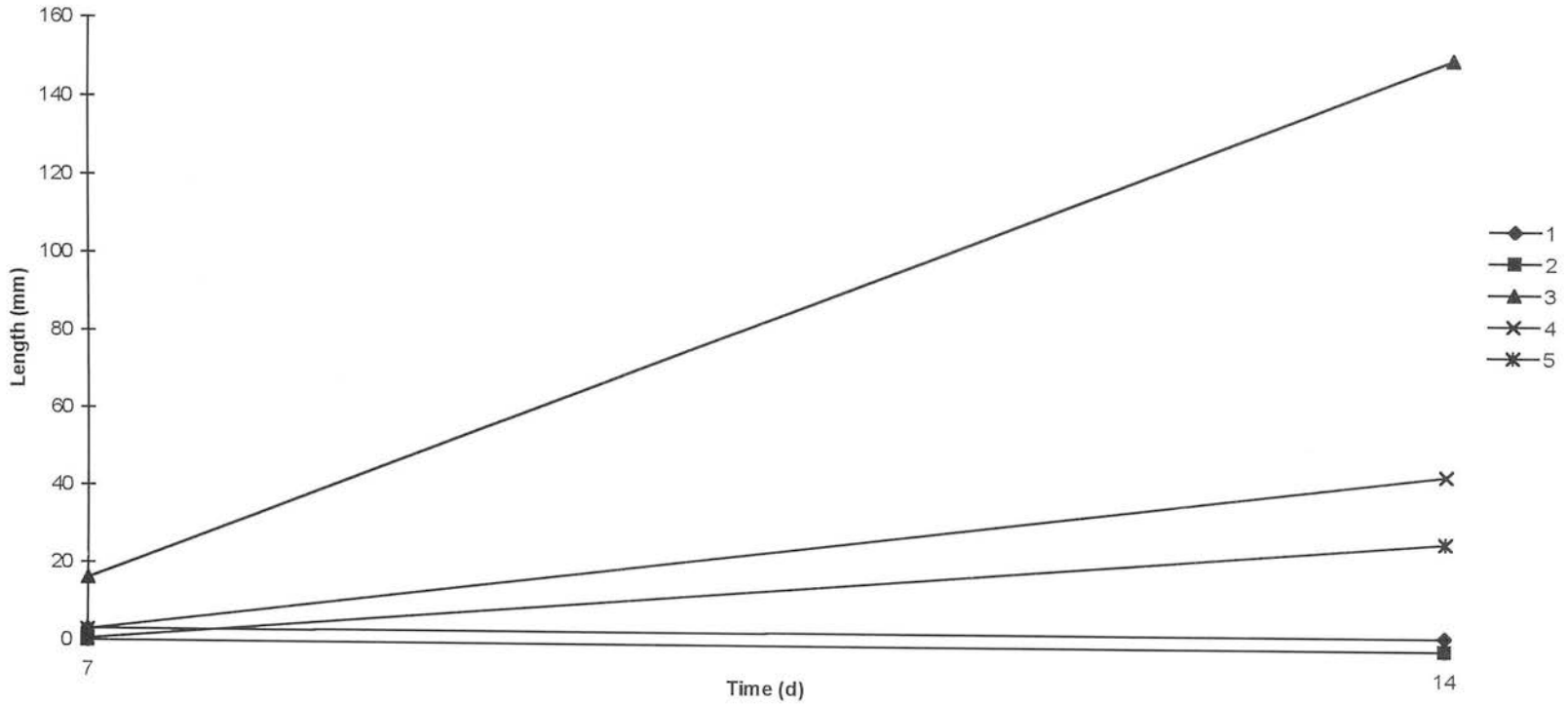

Figure A3.3b: Growth rates (mm/day) of individual replicates in the presence of host exudates

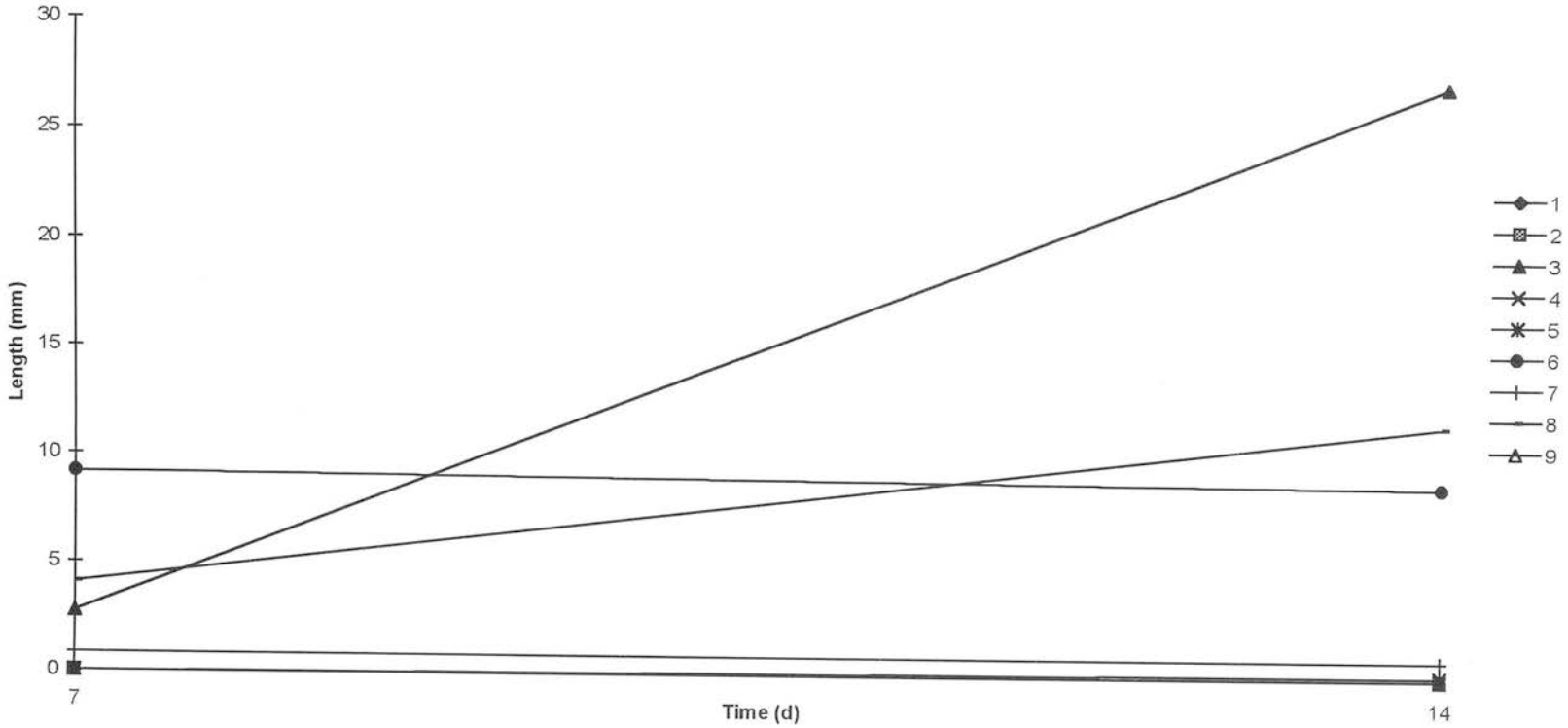

Figure A3.3c: Growth rates (mm/day) of individual replicates in the presence of non-host exudates

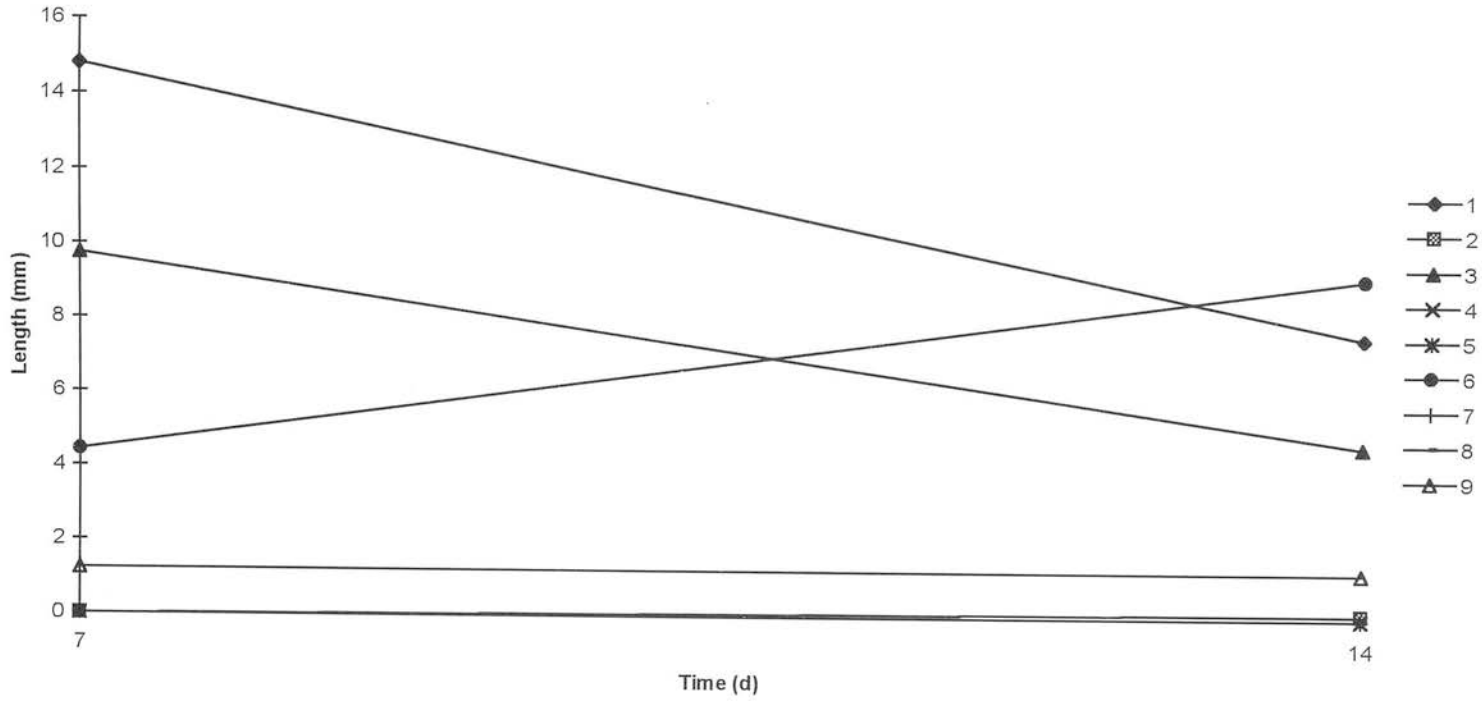

Figure A3.3d: Growth rates (mm/day) of individual replicates in the presence of the flavonoid hesperetin

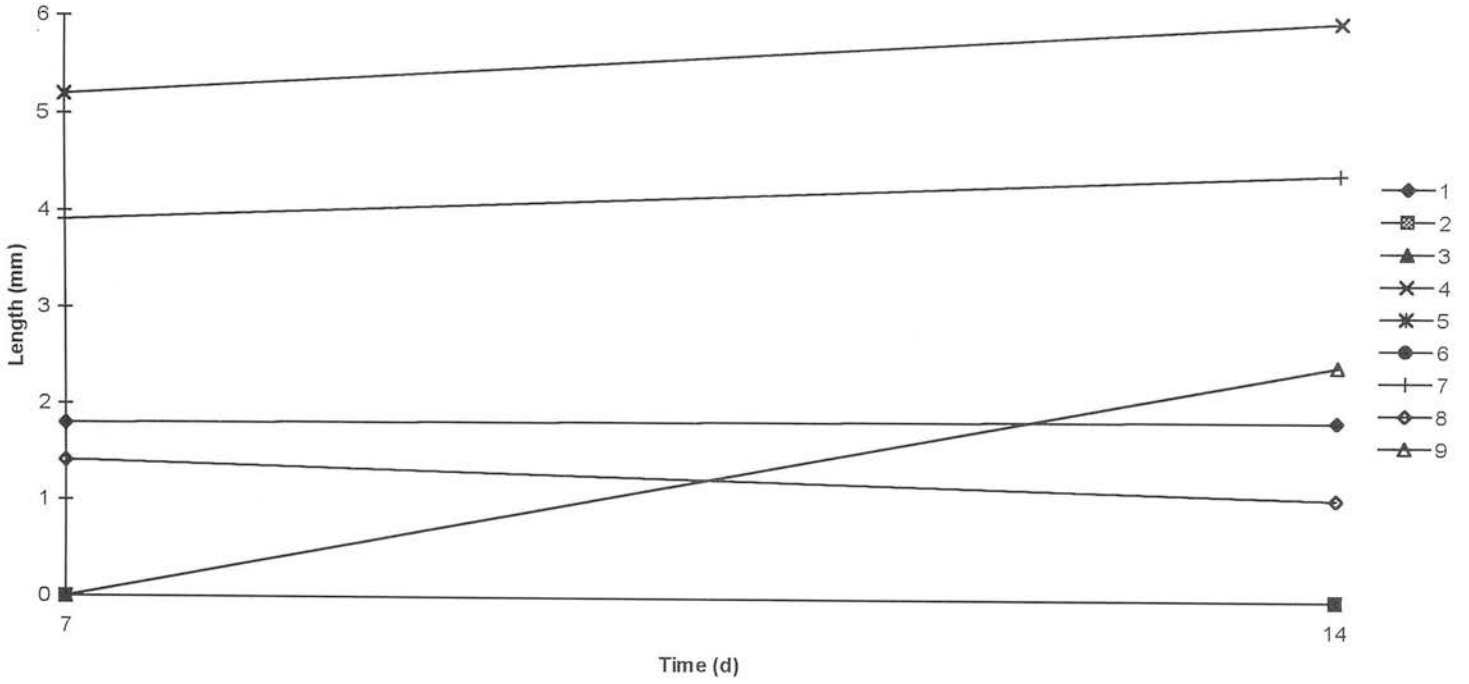

Figure A3.3e: Growth rates (mm/day) of individual replicates in the presence of the flavonoid naringenin

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